Isolation and Partial Characterization of Microorganisms from Leather Industry Wastewater

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List of Figures

1. Figure 1:Role of Microorganisms in Heavy Metal Bioremediation (media/image3.png)
2. Figure 2:Microbial Diversity and Ecological Relevance (media/image2.png)
3. Figure 3: Antibiotic Susceptibility Test Results
4. Figure 4: Colony Morphology of Zn-Resistant Isolates
5. Figure 5:Indole Test Results
6. Figure 6: Urease Test Results
7. Figure 7: Nitrate Reduction Test Results
8. Figure 8: Catalase Test for Zn-Resistant Isolate
9. Figure 9: Oxidase Test Results
10. Figure 10: Motility Test for Zn-Resistant Isolate
11. Figure 11:Motility Test for Cr/Cd/Ni-Resistant Isolates
12. Figure 12: Gram Staining of Zn-Resistant Isolate
13. Figure 13: Gram Staining of Cr/Cd/Ni-Resistant Isolates
14. Figure 14: Sugar Fermentation Test Results
15. Figure 15:Molisch Test for EPS Production
16. Figure 16: Barfoed Test for EPS Production
17. Figure 17: Benedict Test for EPS Production
18. Figure 18: Iodine Test for EPS Production
19. Figure 19: Congo Red Agar Screening for EPS

List of Tables

1. Table 1: Biochemical Characterization of Bacterial Isolates
2. Table 2: MIC Determination Using 100 mg/mL Stock Solution
3. Table 3:MIC Determination Using 10 mg/mL Stock Solution
4. Table 4: Antibiotic Susceptibility Test Results
5. Table 5:Biochemical and CRA Screening for EPS Production
6. Table 6:Reducing Sugar Quantification (Sample 1)
7. Table 7: Reducing Sugar Quantification (Sample 2)
8. Table 8:Pigmentation Production Under Metal Stress
9. Table 9: UV-Vis Spectroscopy Analysis of Microbial Pigments

Abbreviation

Abbreviation	Full form
ATGs	Antibiotic Resistance Genes
AST	Antibiotic Susceptibility Test
ATP	Adenosine Triphosphate
BOD	Biochemical Oxygen Demand
CLSI	Clinical and Laboratory Standards Institute
CRA	Congo Red Agar
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9
CETPs	Common Effluent Treatment Plants
DNS	3,5-dinitrosalicylic acid method
EPS	Exopolysaccharides
HGT	Horizontal Gene Transfer
HMRGs	Heavy Metal Resistance Genes
MFC	Microbial Fuel Cell
MIC	Minimum Inhibitory concentration
NADH	Nicotinamide Adenine Dinucleotide
PCR	Polymerase Chain Reaction
SDG	Sustainable Development Goals
SMEs	Small- and Medium-sized Enterprises
UV-Vis	Ultraviolet-Visible spectrophotometry

Abstract

The pollution of the environment with heavy metals originating from leather tannery effluents has become a major environmental problem because metal is toxic, non-biodegradable, and toxic to both organisms and the environment, especially zinc (Zn), chromium (Cr), cadmium (Cd), and nickel (Ni). This work aimed to isolate and characterize heavy metal-resistant bacteria from tannery wastewater for possible use as bioremediation agents. Isolates were grown on nutrient-agar with or without metal supplementation. Bacterial strains were tested for its tolerant capacity using Minimum Inhibitory Concentration (MIC) technique by serial dilution.

Detailed biochemical analysis including catalase, oxidase, urease, indole, Nreduction, motility and sugar fermentation tests were also done. The nature of the cell wall was examined by Gram staining. Antimicrobial susceptibility testing using chloramphenicol and tetracycline was useful in examination of putative co-resistance mechanisms.Exopolysaccharide (EPS) production (critical for biofilm formation and environmental persistence) was proved by Molisch's test and Congo Red Agar. Metabolic activity, as reflected by reducing sugar, was measured using the DNS method. Metal stress-dependent pigment formation was detected and pigments were analyzed by UV-Visible spectrophotometry.

This investigation brings light to the adaptive responses and metabolic features of metal-resistant bacteria and amplifies the knowledge of their importance to natural detoxification processes and their potential for the development of sustainable wastewater treatment processes

Keywords:-
Bioremediation, MIC, Antibiotic Resistance,
UV vis spectrophotometry, Exopolysaccharides (EPS)

CONTENT

	Page no.
Title Page ……	1
Completion Certificate ……	2
Plagiarism Declaration ……	3
Msc Certificate ……	4
Acknowledgement ……	5
List Of Figures …….	6
List Of Tables ……	7
Abbreviation …….	8
Abstract ……	9
Chapter 1 ……..	11
Introduction …….	11
1.1 Background And Context …….	11
1.2 Problem Statement ……	12
1.3 Role Of Microorganisms In Heavy Metal Bioremediation ……	13
1.4 Microbial Diversity And Ecological Relevance ……	14
1.5 Aims & Objectives …….	16
Chapter – 2 ……..	17
Literature Review …….	17
Chapter -3 ……	25
Materials And Methods ……	25
3.1 Materials Required ……	25
3.2 Sample Collection ……	25
3.3 MIC Determination ……..	25
3.4 Biochemical characterization …….	25
3.5 Antibiotic Susceptibility Test ……	26
3.6 Microbial Characterization By Using 16srrna Sequencing ……	27
3.7 EPS Production & Biochemical Characterization ……	28
3.8 Pigment Production In Heavy Metal Resistant Bacterial Culture ……	28
3.9 Uv Vis Analysis Of Microbial Pigments ……	29
Chapter 4 ……	31
Result …….	31
Colony Morphology ……..	31
MIC Determination ……	32
Biochemical characterization ……	33
Antibiotic Susceptibility Test ……	35
EPS Production & Biochemical Characterization ……..	36
Microbial Characterization By Using 16 srrna Sequencing ……	40
Pigment Production In Heavy Metal Resistant Bacterial Culture …….	50
UV VIS Analysis Of Microbial Pigments ……	51
Chapter 5 …….	53
Discussion ……	53
Conclusion ……..	56
Reference ……	57
Appendix ……	60
Plagiarism Report ……	63

Chapter 1
Introduction

1.Background and Context

The leather tanning industry is a significant contributor to the global economy, particularly in countries like India, which is among the top producers and exporters of leather products. Cities such as Kanpur, Tamil Nadu, and Kolkata host numerous tanneries that provide employment and contribute to industrial growth. However, the environmental impact of leather processing is substantial. The tanning process involves the use of various chemicals, including chromium pollutant salts, which are essential for stabilizing the collagen fibers in hides. This process generates large volumes of wastewater containing high concentrations of heavy metals such as nickel, zinc, lead, and cadmium (Cd).along with other pollutants.

The leather tanning industry, valued at over $400 billion globally, is a vital economic driver in countries like India, Bangladesh, Italy, and Ethiopia. But its impact on the environment is astounding: roughly80% of processed raw materials are discharged as waste, releasing 300–400 mg/L of chromium (Cr) and other toxic metals into ecosystems annually (Ayele et al., 2021; Likhaw Tadesse, 2017). Tannery effluents contain high concentrations of hexavalent chromium (Cr^{6+}) , cadmium (Cd), lead (Pb), zinc (Zn), and nickel (Ni), which persist in water and soil due to their non-biodegradable nature., Cr^{6+}a primary tanning agent, is classified as a Group 1 carcinogen by the IARC, linked to lung cancer, renal failure, and DNA damage (Kapahi & Sachdeva, 2019). Secondary pollutants like chromium-laden sludge contaminate agricultural lands, entering the food chain through bioaccumulation in crops such as rice and wheat (Wahyu Nugraha et al., 2018).

Tannery effluents are characterized by high Total dissolved solids, chemical oxygen demand (COD), and biochemical oxygen demand (BOD), and significant levels of toxic heavy metals. These pollutants are often discharged into nearby water bodies or landfills without adequate treatment, leading to severe environmental contamination (Likhaw Tadesse, 2017). The non-biodegradable nature permits the persistence of heavy metals in the environment, where they canaccumulate in the soil and water, entering the food chain and posing serious health risks to humans and animals.

2. Problem statement

The leather tanning industry is one of the most polluting sectors globally, with wastewater being a major environmental concern due to the discharge of toxic materials, particularly heavy metals as lead, chromium, cadmium, and nickel. In many developing countries, including India, the situation is exacerbated by the absence or poor enforcement of environmental regulations, limited technological infrastructure, and inadequate investment in wastewater treatment facilities. Consequently, significant amounts of untreated or inadequatelytreated tannery effluents are routinely released into surface waters, open land, and drainage systems. This indiscriminate disposal not only affects the quality of natural resources but also poses long-term ecological and public health risks. (Wang et.al, 2021)

The chemical oxygen demand (COD) of these effluents is usually high, biological oxygen demand (BOD), total dissolved solids (TDS), and contain toxic concentrations of persistent heavy metals(Pande et.al., -2022)Chromium, especially in its hexavalent form (Cr(VI)), is the most dominant pollutant associated with tanning processes. Once released into the environment, these heavy metals do not degrade and tend to bioaccumulate in soil, water bodies, crops, and eventually in humans through the food chain, leading to toxic effects such as organ failure, neurological disorders,carcinogenicity, and teratogenicity.(kapahi &Sachdeva, 2019).

To manage this growing problem, industries have traditionally relied on physicochemical methods for effluent treatment. These methods include chemical precipitation, ion exchange, adsorption, electrochemical treatments, and membrane filtration (e.g., reverse osmosis). While effective in certain scenarios, these approaches come with significant drawbacks. Firstly, they are energy- and capital-intensive, requiring sophisticated machinery and skilled personnel, which makes them less feasible for small- and medium-sized enterprises (SMEs), especially in resource-limited settings (Basu et.al.,1997).Secondly, they often result in the production of toxic sludge or secondary pollutants, which require additional processing and disposal, making the waste management chain more complex.Moreover, these methods are not always efficient in treating effluents with low levels of complicated combinations or heavy metals, which are frequently seen inreal-world tannery discharges(.Thatoi et.al., 2014)

Additionally, many treatment plants, including Common Effluent Treatment Plants (CETPs), face operational inefficiencies, overloading, and poor maintenance due to financial constraints, lack of skilled personnel, and bureaucratic delays. Plants in certain areas run at or below capacity.leading to the direct discharge of untreated effluents. Consequently, nearby communities, agricultural fields, and aquatic ecosystems become increasingly exposed to persistent heavy metal pollution(.Li et. al, 2017)

In light of these constraints, it is imperative to investigate other,sustainable, and cost-effective remediation strategies. Bioremediation — Utilizing microorganisms to cleanse or remove contaminants offers a promisingsolution.(Ayele et.al.,2021)Unlike physicochemical methods, microbial treatment is eco-friendly, less costly and frequently suitable for in situ uses.Some microorganismsspecies, especially those isolated from heavy metal-contaminated environments, possess natural resistance mechanisms that enable them to tolerate, immobilize, or transform toxic metals into less harmful forms. However, the

successful implementation of such biological treatments depends on identifying and characterizing robust microbial strains that are not only resistant to high levels of heavy metals, but they are also effectively eliminated from wastewater matrices. (Wuhayu Nugruha, 2018)

Traditional methods for heavy metal removal—chemical precipitation, ion exchange, and reverse osmosis—are energy-intensive, cost-effective ($50–$200 per ton of wastewater), and generate hazardous sludge (Ayele et al., 2021). For example, chemical precipitation with sulfides or limeproduces voluminous sludge requiring secure landfill disposal, which accounts for 30–40% of operational costs (Budi Kurniawan et al., 2022). These methods also fail to address mixed-metal contamination, a hallmark of tannery waste.

Therefore, the current environmental and technological challenges demand a paradigm shift towards microbial-based treatment approaches, necessitating scientific investigations into the isolation, resistance profiling, and functional characterization of indigenous heavy metal-tolerant microorganisms from tannery-polluted environments(Ayele&Gadeto, 2021)

3.Role of Microorganisms in Heavy metal Bioremediation

Heavy metals' toxicity and persistence in industrial effluents, especially from theleather tanning industry, poses a formidable challenge to environmental sustainability and public health. Traditional remediation techniques, while partially effective, often fail to offer a comprehensive solution due to high costs, energy demands, and generation of secondary waste. On the other hand, bioremediation, which uses biological agents, especially microbes, to break down, change, or immobilize pollutants in the environment,presents a promising, eco-conscious, and cost-effective alternative for mitigating heavy metal pollution.

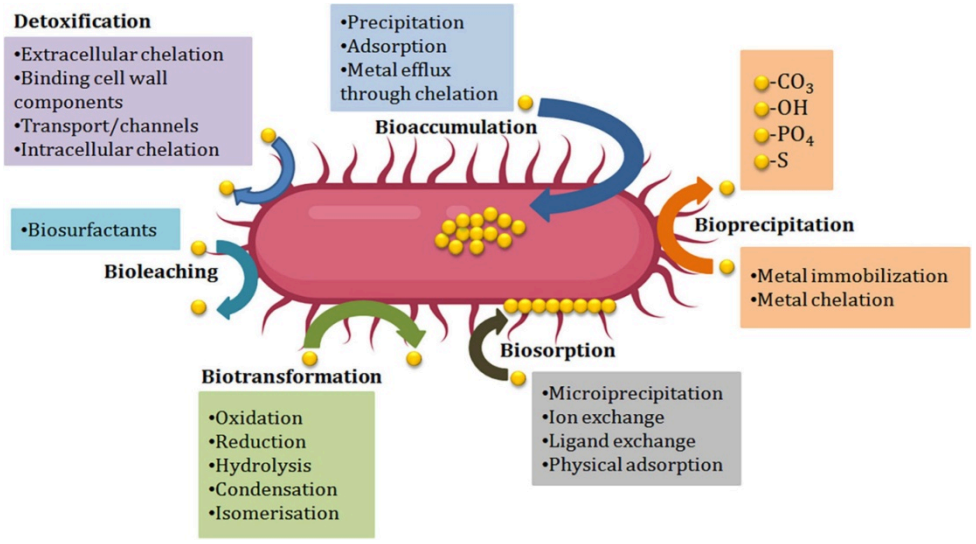


Figure 1:- Role of Microorganisms in Heavy Metal Bioremediation

Microorganisms, including bacteria, fungi, algae, and archaea, have evolved remarkable adaptive mechanisms to survive and thrive in environments contaminated with high concentrations of heavy metals. These organisms are often isolated from polluted sites such as industrial wastewater, mining sites, and tannery waste canals, where prolonged exposure to heavy metals has driven the development of unique genetic and biochemical resistance strategies. These adaptations enable them not only to tolerate heavy metals but also to accumulate, sequester, or detoxify them, thus playing a crucial role in bioremediation.

Living or dead microbial biomass binds heavy materials passively through a process called biosorption.metal ions onto their cell surfaces through physicochemical interactions. The functional groups found in the microbial cell wall, including phosphate, carboxyl, hydroxyl, and amino groups — have a high affinity for metal ions. This allows microorganisms to remove heavy metals from aqueous solutions efficiently. Biosorption does not require metabolic activity and can occur in both viable and non-viable cells, making it highly advantageous for wastewater treatment.

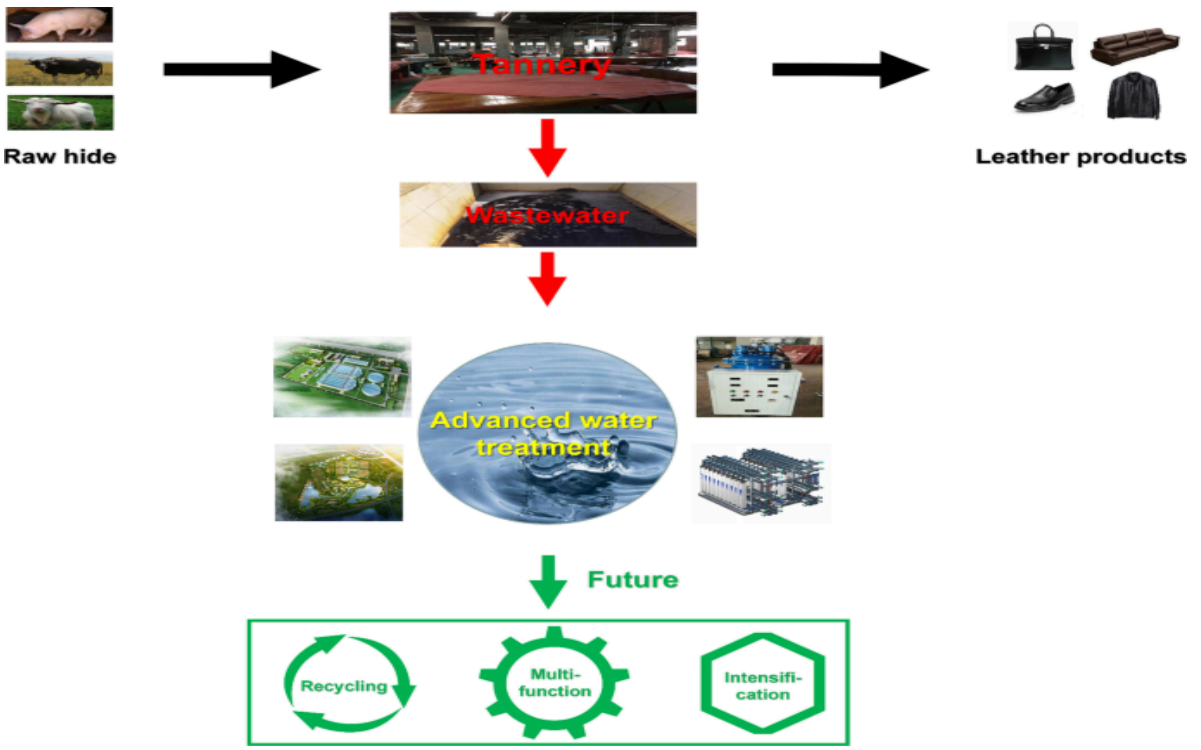
In contrast to biosorption, bioaccumulation is an active, energy-dependent process wherein metal ions are transported across the cell membrane and accumulate inside the cytoplasm or organelles. This internal sequestration helps maintain cellular homeostasis and prevent toxic damage. While this process can be slower than biosorption, it is often more selective and can contribute to long-term immobilization of heavy metals.

Microbial Diversity and Ecological Relevance

A wide range of microorganisms have been documented for their heavy metal resistance and bioremediation potential. Bacterial genera such asPseudomonas,Bacillus,Rhodococcus, Achromobacter, Micrococcus, and Enterobacter, as well as fungal genera likeAspergillus and Penicillium, have

demonstrated capabilities for tolerating and removing heavy metals from industrial effluents. These organisms often display multi-metal resistance and can improve their remedial efficacy in field settings by working together in groups.

Another significant benefit of microbial bioremediation is its versatility. In contrast to conventional systems that require specific environmental conditions, microorganisms can operate under diverse pH, temperature, and salinity ranges. Their ability to form biofilms and survive in extreme environments enhances their resilience and long-term effectiveness in treating wastewater. Furthermore, microbial bioremediation can be integrated with other treatment methods to improve the overall removal efficiency, like artificial wetlands or phytoremediation.



Aims :-

To isolate, characterize, and evaluate heavy metal-resistant bacteria from leather tannery wastewater for their potential application in bioremediation.

Objectives :-

1. To isolate bacterial strains from tannery wastewater capable of growing in the presence of heavy metals such as Cr^{6+}, Zn^{2+}, Cd^{2+}, Ni^{2+}.

2. To determine the Minimum Inhibitory Concentration (MIC) of selected heavy metals for each isolate using serial dilution methods.

3. To characterize the isolates biochemically through tests such as indole, urease, nitrate reduction, catalase, oxidase, motility, and sugar fermentation.

4. To perform Gram staining for morphological classification of the bacterial isolates.

5. To assess antibiotic susceptibility of the metal-resistant isolates using chloramphenicol and tetracycline.

6. To evaluate exopolysaccharide (EPS) production using Congo Red Agar and biochemical tests, to explore their role in biofilm formation.

7. To quantify reducing sugar production by the bacterial isolates using the DNS method.

8. To observe and analyze pigment production under metal stress using UV-Visible spectrophotometry to explore microbial metabolic responses.

Chapter - 2
Literature Review

Isolation of heavy metal resistant organisms from leather tannery waste

The leather tanning industry, a global economic powerhouse valued at over $400 billion, generates significant environmental degradation through heavy metal pollution. Approximately 80% of processed raw materials are discharged as waste, releasing 300–400 mg/L of chromium (Cr) annually, alongside cadmium (Cd), lead (Pb), zinc (Zn), and nickel (Ni) into ecosystems (Ayele & Godeto, 2021; Likhaw Tadesse, 2017). Hexavalent chromium (Cr^{6+}), a Group 1 carcinogen, persists in water and soil, contaminating agricultural crops such as rice and wheat, thereby entering the food chain and posing severe health risks, including lung cancer and renal failure (Kapahi & Sachdeva, 2019; Wahyu Nugraha et al., 2018). Conventional remediation methods like chemical precipitation and ion exchange are not only costly ($50–$200 per ton of wastewater) but also generate hazardous sludge, failing to address mixed-metal contamination (Ayele et al., 2021; Budi Kurniawan et al., 2022). This has driven interest in microbial bioremediation, a sustainable approach leveraging metal-resistant microorganisms to detoxify pollutants while aligning with global sustanablet Goals (SDG 6 and 12).

Microorganisms employ diverse mechanisms to counteract metal toxicity. Bacteria such as *Acinetobacter johnsonii* and *Brevundimonas terrae*, isolated from tannery sludge, tolerate Cr concentrations up to 1,000 mg/L through plasmid-borne *chrA* and *czcCBA* operons encoding efflux pumps

(Bonilla-Espadas et al., 2024; Pande et al., 2022). *Pseudomonas fluorescens* reduces Cr^{6+} to less toxic Cr^{3+} via soluble chromate reductases with 95% efficiency under aerobic conditions, while *Bacillus safensis* bioaccumulates Pb as intracellular phosphate granules, by 80%it reduced the bioavailability (Kapahi & Sachdeva, 2019; Tamil Selvi et al., 2012). Engineered strains like *Escherichia coli* express metallothioneins to sequester Cd and Pb with 70% efficiency, showcasing the potential of genetic engineering in enhancing bioremediation (Wang et al., 2021). Fungi also play critical roles: *Aspergillus niger* achieves 70–90% Cr biosorption via chitin and melanin in cell walls, with a maximum uptake capacity of 180 mg/g, while *Trichoderma harzianum* immobilizes Cr^{6+} through glutathione-dependent redox reactions (Antonia Cruz-Hernandez et al., 2022; Kassim Ghaima et al., 2018). Molecular resistance mechanisms include efflux systems like CzcA in *Cupriavidus metallidurans*, which expels Zn, Cd, and Co using proton gradients, and enzymatic detoxification via chromate reductases in *Bacillus subtilis* (Nies, 2003; Thatoi et al., 2014).

Isolation of metal-resistant organisms relies on strategic sampling from high-contamination sites such as Vellore, India (Cr: 2,500 mg/kg), Modjo, Ethiopia (Cd: 45 mg/L), and Naples, Italy (Zn: 1,200 mg/kg) (Bonilla-Espadas et al., 2024; Likhaw Tadesse, 2017). Selective media supplemented with metals (e.g., Cr: 500 mg/L) and chromium-reducing media with phenol red indicators enable the cultivation of resistant strains (CLSI, 2021; Thatoi et al., 2014). Phenotypic characterization through minimum inhibitory concentration (MIC) assays reveals *Pseudomonas putida* tolerating Zn (1,200 mg/L) and Cd (800 mg/L), while *Aspergillus niger* withstands Cr up to 2,500 mg/L (Kapahi & Sachdeva, 2019; Tamil Selvi et al., 2012). Genomic techniques such as 16S rRNA sequencing and metagenomics can detect known and unculturable species, for instance, Geobacter in anaerobic sludge (Bonilla-Espadas et al., 2024; Hao et al., 2020). However, co-resistance dilemma remains due to the simultaneous presence of heavy metal resistance genes (HMRGs) and antibiotic resistance genes (ARGs) on mobile genetic elements, such as czaA (metal efflux) and sul1 (sulfonamide resistance) genes carried on Klebsiella pneumoniae (Li et al., 2017).

Despite its promise, industrial adoption faces barriers. Lab-scale bioreactors achieve 90% Cr removal, but scaling to 10,000-L systems reduces efficiency to

50–60% due to biomass washout (Budi Kurniawan et al., 2022). Microbial consortia cost $12–$15 per kg of sludge treated, exceeding chemical methods ($8/kg), though innovations like alginate-immobilized *Bacillus subtilis* and biochar-enhanced *Trichoderma* biosorption improve cost-effectiveness and reusability (Thatoi et al., 2014; Antonia Cruz-Hernandez et al., 2022). Genetic engineering breakthroughs, such as CRISPR-Cas9-modified *Pseudomonas* for arsenic hyperaccumulation and synthetic *Shewanella oneidensis* biofilms for simultaneous Cr^{6+} reduction and energy generation, highlight cutting-edge advancements (Hao et al., 2020; Wang et al., 2021). Hybrid systems integrating phytoremediation (e.g., sunflowers) with microbial consortia reduce soil Pb by 65%, demonstrating synergistic potential (Abd Elnabi et al., 2023). Future efforts must prioritize omics-driven discoveries to identify stress-responsive genes, policy reforms to incentivize bioremediation, and community engagement to implement low-cost biofiltration techniques.

Industrial activities, such as leather production, are significant contributors to environmental pollution, releasing hazardous heavy metals like chromium and organic waste into ecosystems, which pose risks to both environmental and human health (Bonilla-Espadas et al., 2024; Verma & Sharma, 2020). Concurrently, chronic infections, particularly in wound care, are increasingly complicated by biofilm-forming bacteria that exhibit heightened antibiotic resistance (Harika et al., 2020). These interconnected challenges highlight the dual role of microbial communities as both environmental remediators and clinical threats. Recent studies have explored microbial diversity in bioremediation, biofilm dynamics in chronic infections, and advanced methodologies for analyzing microbial ecosystems, offering insights into cross-disciplinary solutions for pressing environmental and public health issues.**

Tannery wastewater and solid waste host diverse microbial communities capable of degrading complex pollutants. For instance, bacteria isolated from tannery wastewater, including *Acinetobacter*, *Brevundimonas*, and *Mycolicibacterium*,* demonstrate dual capabilities for leather biodegradation and chromium resistance (Bonilla-Espadas et al., 2024). These organisms employ enzymatic pathways such as serine proteases and metalloproteases to break down collagen and reduce toxic hexavalent chromium [Cr(VI)] to the less

harmful trivalent form [Cr(III)] (Bonilla-Espadas et al., 2024). Metagenomic analyses of tannery waste further reveal a dominance of **Proteobacteria* (e.g., *Pseudomonas*, *Bacillus*), *Firmicutes*, and *Bacteroidetes*,* with functional profiling identifying proteases as critical enzymes for degrading proteinaceous materials like keratin and collagen (Verma & Sharma, 2020). However, the efficiency of biodegradation varies significantly depending on the tanning agents used. Chrome-tanned leather, for example, shows minimal degradation (7.6% over 33 days), while oxazolidine- and aluminum-tanned variants degrade up to 59.4% and 23.2%, respectively (Bonilla-Espadas et al., 2024). These disparities underscore the inhibitory effects of heavy metals like chromium on microbial activity, necessitating the use of strains with both degradative and metal-resistant traits (Verma & Sharma, 2020). Microbial consortia in tannery environments adapt to pollutants through metabolic pathways such as carbohydrate metabolism and amino acid degradation, yet challenges persist in optimizing these systems for large-scale bioremediation (Bonilla-Espadas et al., 2024; Verma & Sharma, 2020).

In medical settings, biofilms, which are complex communities of microorganisms embedded in extracellular matrices, are responsible for chronicity of infections and increased resistance to antibiotics (Harika et al., 2020). An investigation on 92 bacterial isolates obtained from chronic wounds revealed that 78.2% were biofilm producers and *Staphylococcus aureus* and *Klebsiella pneumoniae* were the most common pathogens (Harika et al., 2020). Biofilm forming isolates displayed significantly greater resistance to antibiotics like ciprofloxacin (89% in S. aureus) and meropenem (71% in Gram negative bacteria) due to delayed penetration of antimicrobials and horizontal gene transfer inside the biofilm matrix (Harika et al., 2020).Among the qualitative methods the Tissue Culture Plate (TCP) with a sensitivity of 47.2–56.9% was the most reliable technique to determine biofilm formation followed by the Tube Method and Congo Red Agar (Harika et al., 2020). These results underscore the importance of comprehensive biofilm screening in clinical microbiology to guide therapeutic approaches and prevent chronic infections.

Recent developments in microbial community analysis, particularly metagenomics, have transformed the investigation of environmental and clinical microbiomes. Belikova & Pham, 2020).For instance, Illumina sequencing of tannery waste metagenome has lead to the identification of thousands of protease encoding genes, suggesting the discovery of novel

enzymes suitable for bioremediation (Verma & Sharma, 2020). Likewise, common assays such as ISO 20136:2020 (4) have been used to measure degradation efficiency and correlate changes in microbial community composition with the different substrate utilization phases (Bonilla-Espadas et al., 2024).Despite these innovations, challenges remain, including biases in PCR amplification and gaps in reference databases, which limit the resolution of metagenomic studies (Verma & Sharma, 2020). Cultivation-based methods, such as the selective isolation of heavy metal-resistant strains, remain indispensable for validating metagenomic predictions and elucidating the functional roles of specific taxa (Bonilla-Espadas et al., 2024).

The intersection of environmental and clinical microbiology reveals intriguing overlaps. *Acinetobacter* species, often found in tannery waste due to their useful role in bioremediation, are also well-known for causing hospital-acquired infections (Bonilla-Espadas et al., 2024; Harika et al., 2020).This duality underscores the risk of resistance gene dissemination between environmental and clinical settings, necessitating a One Health approach to microbial management. Future research should prioritize mechanistic studies to unravel the molecular pathways underlying heavy metal resistance and collagen degradation, as well as in-situ applications of microbial consortia in real-world bioremediation systems (Bonilla-Espadas et al., 2024; Verma & Sharma, 2020).In clinical contexts, the development of biofilm-targeted therapies, such as enzymatic disruptors or quorum-sensing inhibitors, could enhance the management of chronic wounds (Harika et al., 2020). Integrating multi-omics approaches—combining metagenomics, transcriptomics, and proteomics—offers a promising avenue for deciphering complex microbial interactions in both polluted ecosystems and infected tissues (Verma & Sharma, 2020; Bonilla-Espadas et al., 2024). By bridging insights from environmental and clinical studies, researchers can address the dual challenges of pollution and antibiotic resistance, fostering innovations that benefit both planetary and human health.

Tannery effluents harbor microbial communities capable of degrading pollutants and resisting heavy metals. Tamil Selvi et al. (2012) isolated bacteria from a tannery effluent treatment place chennai, India, identifying *Pseudomonas spp., *Escherichia coli*, *Bacillus spp., Flavobacterium spp., and *Alcaligenes* spp.* as dominant genera resistant to lead (Pb), copper (Cu), zinc (Zn),

chromium (Cr), and mercury (Hg). These isolates exhibited minimum inhibitory concentrations (MICs) ranging from 60 to 80 ppm for metals, with *Pseudomonas* spp. exhibiting the greatest resistance, with 90% of isolates tolerating both lead (Pb) and mercury (Hg).Similarly, Bonilla-Espadas et al. (2024) reported *Acinetobacter , Brevundimonas , and Mycolicibacterium* in tannery wastewater, highlighting their enzymatic pathways (e.g., serine proteases) for collagen degradation and Cr(VI) reduction. Verma and Sharma (2020) corroborated these findings through metagenomic analyses, revealing *Proteobacteria* and *Firmicutes* as key taxa in tannery waste, with proteases critical for degrading keratin and collagen.

The success of bioremediation largely relies on how well microorganisms can adapt to the presence of environmental pollutants.Tamil Selvi et al. (2012) noted that microbial consortia in tannery waste adapt via metabolic pathways like carbohydrate metabolism, aligning with Verma and Sharma's (2020) observations. However, heavy metals like chromium inhibit microbial activity, necessitating strains with dual resistance and degradative traits. For instance, chrome-tanned leather showed minimal biodegradation (7.6% over 33 days) compared to oxazolidine-tanned variants (59.4%) (Bonilla-Espadas et al., 2024), underscoring the need for metal-resistant microbes in bioremediation.

Metagenomics has revolutionized microbial community analysis. Verma and Sharma (2020) identified 5,848–9,633 protease-encoding genes in tannery waste, highlighting the potential for discovering novel bioremediation enzymes.However, limitations such as PCR biases and incomplete reference databases still pose challenges.. In contrast, cultivation-based methods, like those used by Tamil Selvi et al. (2012), remain vital for validating resistance phenotypes. Their use of biochemical assays (e.g., oxidase, catalase) and standardized MIC testing provided actionable insights into metal and antibiotic resistance profiles.

The dual role of microbes as environmental remediators and clinical pathogens necessitates a One Health approach. **Pseudomonas** spp., effective in metal detoxification (Tamil Selvi et al., 2012), are also opportunistic pathogens (Harika et al., 2020). Future research should prioritize:

1. **Mechanistic Studies**: Elucidate molecular pathways of co-resistance (e.g., efflux pumps, plasmid-mediated gene transfer).
2. **In-Situ Applications**: Test microbial consortia from studies like Tamil Selvi et al. (2012) in real-world bioremediation.
3. **Biofilm-Targeted Therapies**: Develop enzymatic disruptors to combat clinical biofilms (Harika et al., 2020).
4.*Multi-Omics Integration**: Combine metagenomics, transcriptomics, and proteomics to unravel microbial interactions (Verma & Sharma, 2020).

Kumar et al. (2018)** identified *mer* operons in *Pseudomonas aeruginosa* strains isolated from tannery sludge, enabling mercury volatilization via the *merA* reductase. This mechanism reduces Hg(II) to less toxic Hg(0), offering bioremediation potential. Similarly, **Patel et al. (2021)** characterized chromium reductase enzymes in *Bacillus subtilis*, which convert Cr(VI) to Cr(III) through NADH-dependent pathways. These findings align with Tamil Selvi et al. (2012), who reported *Pseudomonas* spp. Among the microbial populations identified in the tannery effluent, chromium-resistant strains were found to be predominant.

Zhang et al. (2019) demonstrated the role of extracellular polymeric substances (EPS) in *Acinetobacter baumannii* biofilms for sequestering lead and copper. EPS production, induced under metal stress, enhances microbial survival in polluted environments. This complements (Bonilla-Espadas et al. (2024), who highlighted EPS-mediated metal adsorption in tannery wastewater communities.

The co-occurrence of metal and antibiotic resistance genes (ARGs) in environmental bacteria is well-documented. (Li et al. (2020) conducted a metagenomic analysis of tannery sludge, identifying plasmid-borne *czcA* (cadmium-zinc-cobalt resistance) and *blaTEM* (beta-lactamase) genes on the same mobile genetic element. This mechanism of horizontal gene transfer (HGT) accounts for the findings of (Tamil Selvi et al. (2012) regarding the presence of multidrug-resistant *Alcaligenes* species in tannery effluents.

Gupta et al. (2019) isolated *Stenotrophomonas maltophilia* from chromium-contaminated soil, showing co-resistance to tetracycline and zinc. The study linked efflux pump overexpression (e.g., *smeABC*) to both metal extrusion and antibiotic resistance, mirroring Dhakephalkar and Chopade's (1994) findings in *Acinetobacter

Environmental and Clinical Settings –
Biofilms in contaminated environments serve as reservoirs for resistant pathogens.. **Wang et al. (2022)** analyzed biofilms in tannery effluent pipelines, identifying *Klebsiella pneumoniae* strains with dual resistance to chromium and carbapenems. The biofilm matrix facilitated ARG exchange via conjugation, echoing(Harika et al.'s (2020) clinical findings on chronic wound biofilms.

In clinical contexts, (Singh et al. ,2021) demonstrated that *Staphylococcus epidermidis* biofilms on medical devices exhibit upregulated *mecA* (methicillin resistance) and *copA* (copper resistance) genes under metal stress. This underscores the need for biofilm-targeted therapies, as proposed by Harika et al. (2020)

Innovative approaches to enhance bioremediation include bioaugmentation and phytoremediation. (Fernández et al. ,2023)** engineered *Mycolicibacterium smegmatis* to overexpress chromate reductases, achieving 95% Cr(VI) removal in synthetic tannery wastewater. Similarly, (Ijaz et al. 2022)** combined *Trichoderma harzianum* (fungi) with *Bacillus* spp. to degrade tannery dyes and adsorb heavy metals synergistically.

Joshi et al. (2020) developed a microbial fuel cell (MFC) using tannery sludge isolates (**Shewanella** spp.), generating bioelectricity while reducing Cr(VI). This aligns with (Tamil Selvi et al.'s (2012) emphasis on **Pseudomonas* spp. for industrial applications.

Studies from diverse regions highlight the universality of tannery pollution challenges. (Olaniran et al. 2021) reported arsenic-resistant *Arthrobacter* spp. in Nigerian tanneries, while (Rahman et al. 2019) identified multidrug-resistant *Vibrio* spp. in Bangladeshi tannery effluents. These findings stress the need for

global policies to regulate industrial discharges, as advocated by(Kumar et al. (2018).

Chapter -3
Materials and Methods

3.1 Materials required :-

The materials equipment, media and reagent used in this study are systematically listed in appendix-I.

3.2 *Sample collection :-*

Wastewater samples were obtained from the Kolkata Leather Complex (KLC) in Bantala, West Bengal, India—one of the largest integrated leather processing hubs in South Asia. The samples were gathered from open drainage canals and effluent discharge points connected to tannery units. Collection was conducted using sterile 500 mL polypropylene bottles under aseptic conditions to avoid external contamination. Each sample was immediately labeled with date, time, and location coordinates and transported to the laboratory in an icebox maintained at 4°C. All samples were processed within 4–6 hours of collection for downstream microbial isolation and characterization.

3.3 *Minimum Inhibitory Concentration (MIC) Determination:-*

The MIC for Zn, Cr, Ni, and Cd was determined using a serial dilution method. A stock solution of each heavy metal was prepared at a concentration of 100 mg/mL and then serially diluted.After initial MIC determination, the stock solution was further diluted to 10 mg/mL for more precise analysis. The minimum inhibitory concentration (MIC) refers to the smallest amount of a substance needed to completely stop the bacteria from growing.

3.4 *Biochemical Characterization*

Biochemical tests were conducted to characterize heavy metal-resistant bacterial isolates. The Indole Test was conducted to assess the ability of bacterial isolates to degrade tryptophan into

indole. If the enzyme tryptophanase is present, it catalyzes the hydrolysis of tryptophan, resulting in the production of indole, pyruvate, and ammonia.The test was conducted by inoculating the bacterial culture into tryptone broth and then incubated at 37 °C for 24 to 48 hours to allow for bacterial growth and metabolic activity.After incubation, Kovac's reagent was added..

The Urease Test was conducted to detect the presence of urease enzyme in bacterial isolates.The enzyme urease facilitates the conversion of urea into ammonia and carbon dioxide through hydrolysis. For the urease test, a medium containing urea and phenol red (as a pH indicator) was inoculated with the test organism and incubated at 37 °C

Nitrate reduction test - This test assessed the bacteria's ability to reduce nitrate (NO_3^-) to nitrite (NO_2^-) or beyond, indicating their nitrate reductase activity. Bacterial cultures were inoculated into nitrate broth and incubated at 37 °C for 24–48 hours. Following incubation, nitrate reagents A and B were added to detect the presence of nitrite.After incubation, nitrate reagents A and B were added.

The Catalase Test was conducted to detect the presence of the catalase enzyme, which breaks down hydrogen peroxide (H_2O_2) into water and oxygen. "A small amount of each bacterial isolate was placed on a glass slide, followed by the addition of a few drops of 3% hydrogen peroxide."
The Oxidase Test was performed to detect the presence of cytochrome c oxidase in bacterial isolates. A few drops of oxidase reagent (tetramethyl-p-phenylenediamine) were applied directly to a bacterial colony on filter paper. If no purple or dark blue color appeared within 30 seconds, the result was considered negative, indicating that the bacteria did not produce cytochrome c oxidase.

The Motility Test was conducted to evaluate the movement capability of heavy metal-resistant bacterial isolates. Semi-solid motility agar (0.4%) was prepared, and bacterial cultures were stab-inoculated in the center. The tubes were incubated at 35–37 °C for 24 to 48 hours.

Gram staining was performed to differentiate the bacterial isolates according to their cell wall structure and morphological characteristics.Bacterial smears were prepared on clean glass slides, heat-fixed, and stained using the Gram staining protocol. The staining process involved the sequential application of crystal violet (primary stain), iodine (mordant), alcohol (decolorizer), and safranin (counterstain).

Identifying the Gram reaction of heavy metal-resistant bacteria aids in understanding their structural adaptations, which might influence their resistance mechanisms and ecological roles.

The Sugar Fermentation Test was performed to assess the carbohydrate metabolism of bacterial isolates. The test medium contained a specific sugar (such as glucose or lactose) and a pH indicator (phenol red). Bacterial cultures were inoculated into the medium and incubated at 35–37°C for 24–48 hours.

3.5 *Antibiotic Susceptibility Test (AST)*

The Antibiotic Susceptibility Test (AST) was conducted to evaluate the resistance and susceptibility of heavy metal-resistant bacterial isolates (Zn, Cr, Cd, and Ni) against selected antibiotics—chloramphenicol and tetracycline. The well diffusion method was employed on Mueller-Hinton agar, with antibiotic concentrations of 30, 60, and 80 µL per well. Following incubation at 37 °C for 24 hours, the zones of inhibition were measured to assess the bacterial response. The results revealed varying susceptibility patterns, with some isolates displaying resistance to chloramphenicol while being susceptible to tetracycline, or vice versa. The diameter of inhibition zones increased with higher antibiotic concentrations in some cases, indicating dose-dependent effects. The findings highlight the antibiotic resistance mechanisms of heavy metal-resistant bacteria, contributing to a better understanding of their potential implications in bioremediation and public health.

3.6 Microbial Characteristization by using 16s rRNA sequencing

1. DNA was isolated from the culture provided by the scientist. Its quality was evaluated on 1.0 % agarose gel, a single band of high-molecular weight DNA has been observed.
2. Fragment of 16S rRNA gene was amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel.
3. The PCR amplicon was purified to remove contaminants.
4. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16SrRNA-F and 16SrRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.
5. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software.
6. The 16S rRNA gene sequence was used to carry out BLAST with the ‘nr’ database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix and phylogenetic tree was constructed was constructed using MEGA 11.

3.7 *Exopolysaccharide (EPS) Production and Biochemical Characterization*

The production of exopolysaccharides (EPS) by bacterial isolates was assessed using a combination of biochemical tests and qualitative screening methods. Bacterial cultures were first subcultured in appropriate growth media and incubated at 35–37°C for 24–48 hours. Following incubation, the cultures were centrifuged at 5000 rpm for 10 minutes to separate the supernatant, which contains secreted EPS, from the bacterial pellet.

Biochemical characterization of the EPS was performed using the Molisch, Benedict's, Barfoed's, and Iodine tests.

3.8 *Quantitative Measurement of Reducing Sugars Using DNS Method*

The quantification of reducing sugars in two environmental samples was performed using the 3,5-dinitrosalicylic acid (DNS) method. The samples used in this study were bacterial isolates resistant to heavy metals, specifically Cr, Cd, Ni, and Zn. The objective of the experiment was to estimate the concentration of reducing sugars in these samples to understand their metabolic adaptations.

The DNS method is based on the reduction of DNS by reducing sugars to form a colored complex. To prepare the samples, bacterial cultures were grown in suitable media under optimal conditions for 24–48 hours. Following incubation, the cultures were centrifuged at 5000 rpm for 10 minutes to obtain the supernatant free of bacterial cells.

For the assay, equal volumes (1 mL each) of DNS reagent and the bacterial supernatant were combined."The mixture was vortexed and heated in a boiling water bath for 5–10 minutes to develop the characteristic red-brown color. After cooling to room temperature, the absorbance of the resulting solution was measured at 540 nm using a UV-Visible spectrophotometer. A standard curve was prepared using known concentrations of glucose to calculate the reducing sugar concentration in the samples.

The experiment was conducted with two environmental samples: Sample 1 (comprising Cr, Cd, and Ni-resistant isolates) and Sample 2 (Zn-resistant isolates). Each measurement was performed in triplicate to ensure accuracy. The absorbance values obtained from the spectrophotometer were compared against the glucose standard curve to estimate the concentration of reducing sugars.

3.9 Pigmentation Production in Heavy Metal-Resistant Bacterial Cultures: An Observational Study

The experiment was conducted to observe microbial growth and metabolic activity under different environmental conditions in the presence of heavy metals. The objective was to

assess the adaptive responses of microbial isolates when exposed to chromium (Cr), cadmium (Cd), and nickel (Ni). Test tubes containing sterile nutrient broth were inoculated with microbial cultures resistant to the respective heavy metals.After inoculation, specific concentrations of chromium (Cr), cadmium (Cd), and nickel (Ni) were added to the tubes.". The cultures were then incubated at 35–37°C and monitored at designated time intervals to record growth patterns and metabolic changes.

The experimental setup involved varying environmental conditions, including differences in temperature, aeration, and metal concentrations, to evaluate their effects on microbial behavior. The test tubes were arranged in replicates to ensure accuracy and reproducibility of the results. Observations were recorded after 12 hours and 36 hours of incubation to capture both early and extended responses of the microbes to the metal stress.

After an initial incubation period of 12 hours, the test tubes were visually inspected for changes in turbidity, color, and sediment formation, which could indicate microbial growth, metabolic activity, or interactions with the heavy metals present in the medium. Distinct variations in color and turbidity were noted between the test tubes, suggesting differential microbial responses to the environmental conditions and heavy metal exposure. Preliminary observations indicated that some isolates demonstrated early adaptation, while others showed minimal metabolic changes at this stage.

After 36 hours of incubation, the test tubes were again examined to assess the progression of microbial growth and metabolic activities. More pronounced changes in turbidity, color intensity, and potential biofilm formation were observed, indicating ongoing microbial adaptation and survival mechanisms. The increased color intensity and turbidity in certain test tubes suggested active metabolic processes, possibly including metal resistance, biofilm development, or enzymatic degradation of metal ions. Differences between the microbial responses indicated that the prolonged incubation allowed for the manifestation of adaptive traits, highlighting the resilience of certain isolates under heavy metal stress.

3.10 UV-Vis Spectroscopy Analysis of Microbial Pigments (37°C Sample)

UV-Visible (UV-Vis) spectroscopy was employed to analyze pigment production in the microbial culture. The objective was to characterize the pigment and assess its spectral properties. Microbial cultures were incubated at 37°C, as preliminary observations indicated that this temperature favored the most intense pigmentation. Following incubation for 24–48 hours, the cultures were centrifuged at 5000 rpm for 10 minutes to separate the pigment-containing supernatant from the cell pellet. "Spectrophotometric analysis was performed on the collected supernatant to assess its absorbance properties."

The absorbance spectrum of the pigment extract was recorded using a UV-Vis spectrophotometer. To capture the full visible spectrum and determine the wavelength of maximum absorbance (λmax), absorbance was measured across a range of 380 nm to 700

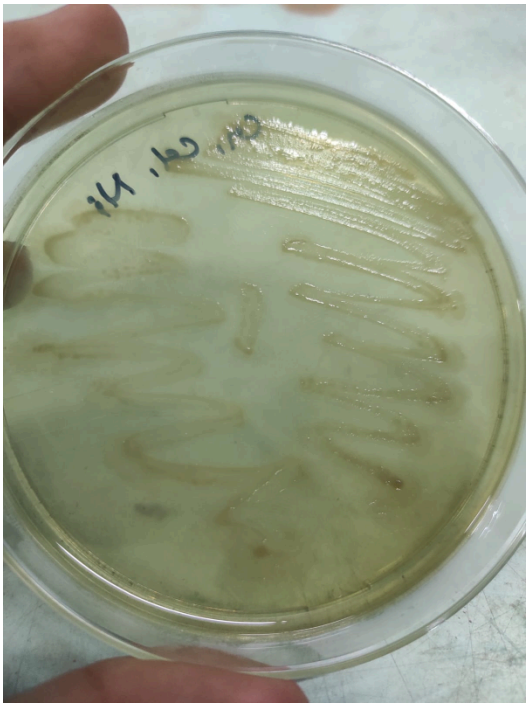
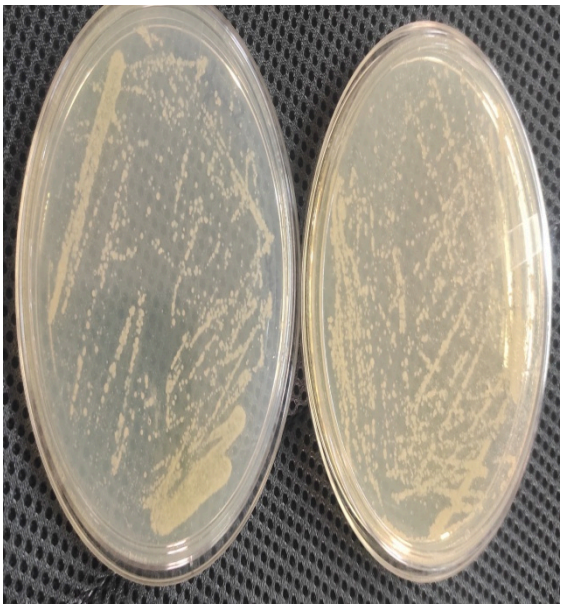
nm. The spectrophotometer was first calibrated using a blank sample containing only sterile culture medium as the reference.”.

Calculation of Absorption Intensity:
To quantify the pigment's light absorption efficiency, the percentage absorption was calculated relative to the highest recorded absorbance value using the following formula:

The calculation was performed for each recorded wavelength to determine the relative absorption efficiency of the pigment. "The wavelength showing the highest absorbance was identified as the λmax."
The resulting absorbance data were plotted to generate an absorption spectrum, highlighting the characteristic peaks. This analysis provided insights into the pigment's optical properties, which could be correlated with its molecular structure. Understanding pigment production and its spectral characteristics is crucial for evaluating the potential biotechnological applications of microbial pigments, including their use as natural colorants and in environmental monitoring.

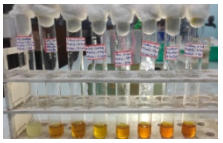
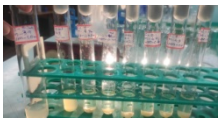
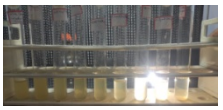
Chapter -4
Results

4.1 Colony morphology

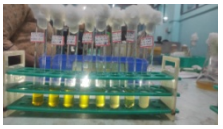
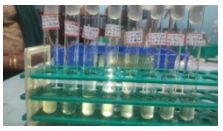
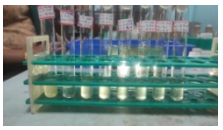
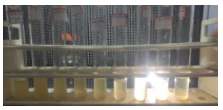
Cr, , Ni resistant	
Zn , Cd resistant Figure -4:- colony morphology of zn and Cd resistant organism	

4.2 *Minimum Inhibitory Concentration (MIC) Determination*

Stock solution -100mg/ml
Solution in each tube - 5ml

Metal	Culture	Media	Volume of metal	
Chromium	0.1ml	4.1ml	0.8ml	
Cadmium	0.1ml	4.7ml	0.2ml	
Zinc	0.1ml	3.9ml	1.0ml	

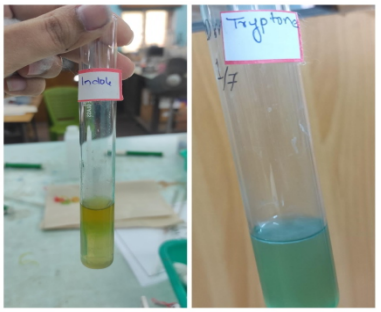
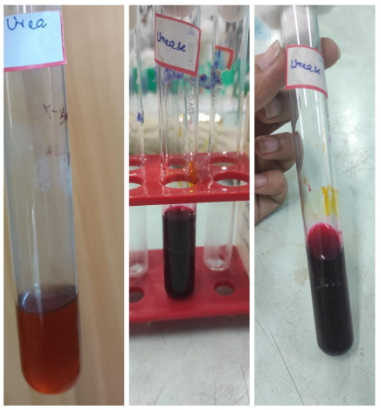
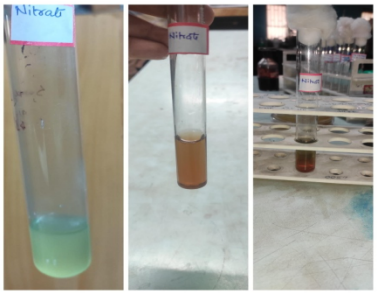
Stock solution - 10mg/ml

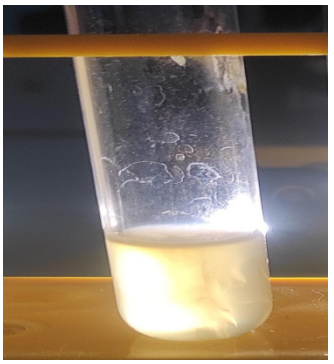
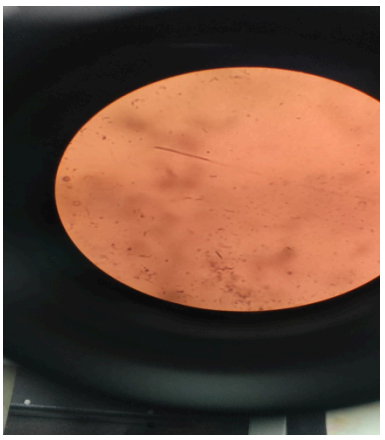
Heavy metal	Culture	Media	Volume of metal	
Chromium	0.1ml	4.7ml	0.2ml	
Nickel	0.1ml	4.3ml	0.6ml	
Cadmium	0.1ml	3.9ml	1.0ml	
Zinc	0.1ml	3.9ml	1.0ml	

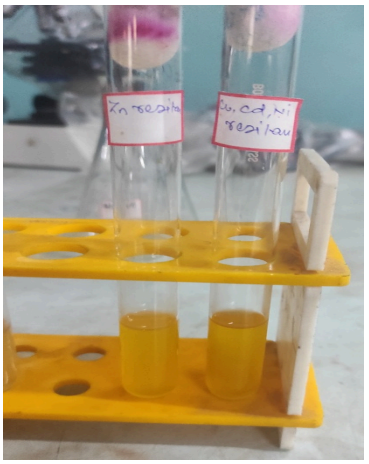
Interpretation of MIC Test

The MIC analysis revealed that the isolated microorganism exhibited significant resistance to multiple heavy metals. The highest tolerance was observed for zinc (up to 20 mg/ml) and chromium (16 mg/ml) from the 100 mg/ml stock solution. At lower concentrations (10 mg/ml stock), the isolate tolerated up to 2.0 mg/ml of cadmium and zinc, 1.2 mg/ml of nickel, and 0.4 mg/ml of chromium. These results indicate a strong metal-resistant phenotype, particularly for zinc and cadmium, suggesting potential for bioremediation applications.

4.3 *Biochemical characterization*

Test	Heavy metal resistant	Result
Indole Fig--5:-	All strains	
Urease	All strains	
Nitrate reduction	All strains	
Catalase	Zn , Cr, Cd, Ni	

Oxidase	All strains	
Motility	Zn, Cr, Cd, Ni resistant isolates	
	Cr, Cd, Ni, Zn resistant isolates	

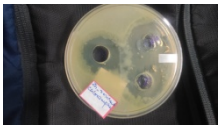
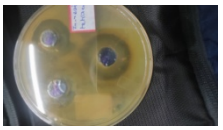
Suger Fermentation	All strains	

Interpretation :-

The Cr isolate is a Gram-negative, non-motile, catalase-negative, oxidase-negative, indole-positive, urease-positive, sugar-fermenting bacterium, with partial nitrate reduction ability.

These traits are partially consistent with some Enterobacteriaceae (e.g., Klebsiella, Shigella, or atypical Proteus), but the catalase-negative result is atypical and should be rechecked.

4.4 *Antibiotic susceptibility Test*

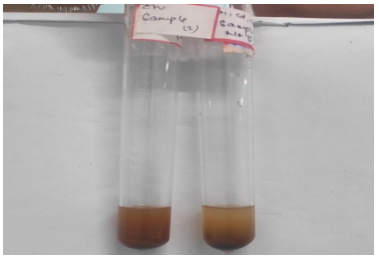
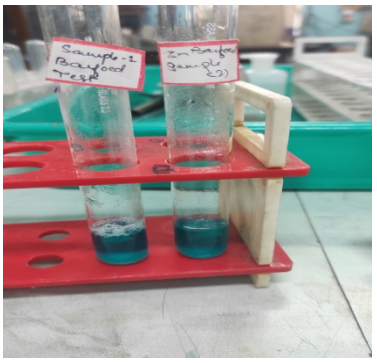
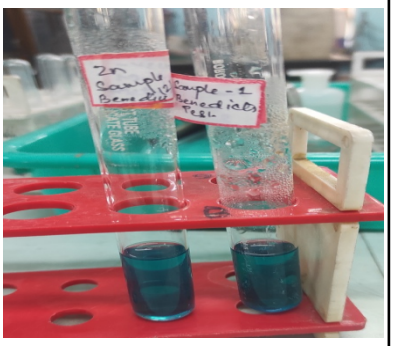
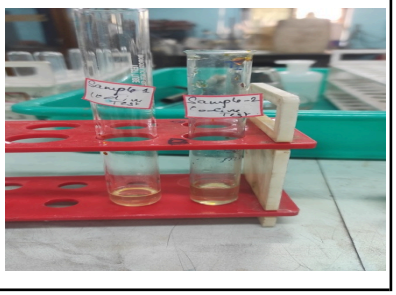
Plate Description	*Antibiotic*	*Inhibitions zone (approx.)*	*Sensitivity*	
Zn. Resistant	Chloramphenicol	*25mm*	Susceptible	
Zn resistant	Tetracycline	~0mm	Resistant	
*Cr, C*d, Ni Resistant	*Chloramphenical*	8-10mm	Intermediate/resistant	
Cr, Cd, Ni resistant	Tetracycline	20mm	Susceptible	

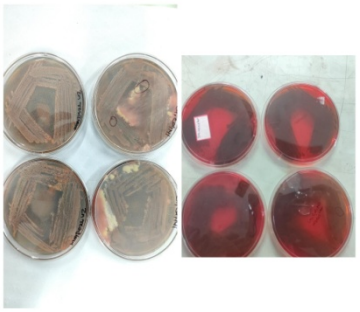
Interpretation
Co-resistance Observed:
All heavy metal-resistant isolates (Zn, Cr, Cd, Ni) exhibit resistance to tetracycline, suggesting a strong correlation between heavy metal resistance and tetracycline resistance. This could be due to co-localization of resistance genes on plasmids or transposons.

Partial Co-resistance to Chloramphenicol:
While Zn-resistant isolates are chloramphenicol-susceptible, Cr-, Cd-, and Ni-resistant isolates show intermediate to full resistance, pointing to possible metal-specific selection pressures influencing antibiotic resistance phenotypes.

Implications for Environmental and Public Health:
These findings suggest that heavy metal contamination in industrial wastewater (e.g., from tanneries) can contribute to antimicrobial resistance (AMR) development in environmental bacterial populations, posing potential risks to human and animal health if such resistant strains disseminate.

4.5 Biochemical characterization, CRA screening in EPS production

Test	Metal resistant	Result
Molisch	All strains	
Barfoed	All strains	
Benedict	All strains	
Iodine	All strains	

Congo Red Agar	All strains	

Interpretation:-

1. EPS Production: The positive CRA test indicates the production of exopolysaccharides (EPS), which plays a key role in the formation of biofilms. This is important in the context of pathogenesis (e.g., in urinary tract infections or biofilm-related infections) and may contribute to the organism's resistance to environmental stresses and antibiotics.

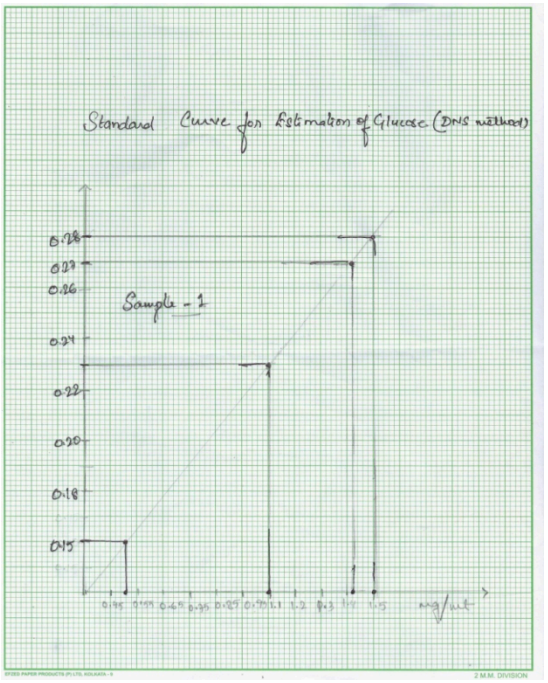
2. Carbohydrate Characteristics:
Molisch Test: The organism produces carbohydrates that may be utilized for energy.
Barfoed and Benedict: The negative results suggest that the isolate does not ferment monosaccharides or reduce reducing sugars, indicating a non-fermentative metabolism.
Iodine Test: The negative iodine test suggests that the organism does not hydrolyze starch, meaning it does not produce amylase or similar enzymes to break down starch.

Possible Organism Identity:

The EPS production and carbohydrate fermentation profile (positive Molisch and negative for sugar reduction) are consistent with certain Gram-negative bacteria like Klebsiella pneumoniae or Pseudomonas aeruginosa, both known for producing EPS and biofilms.

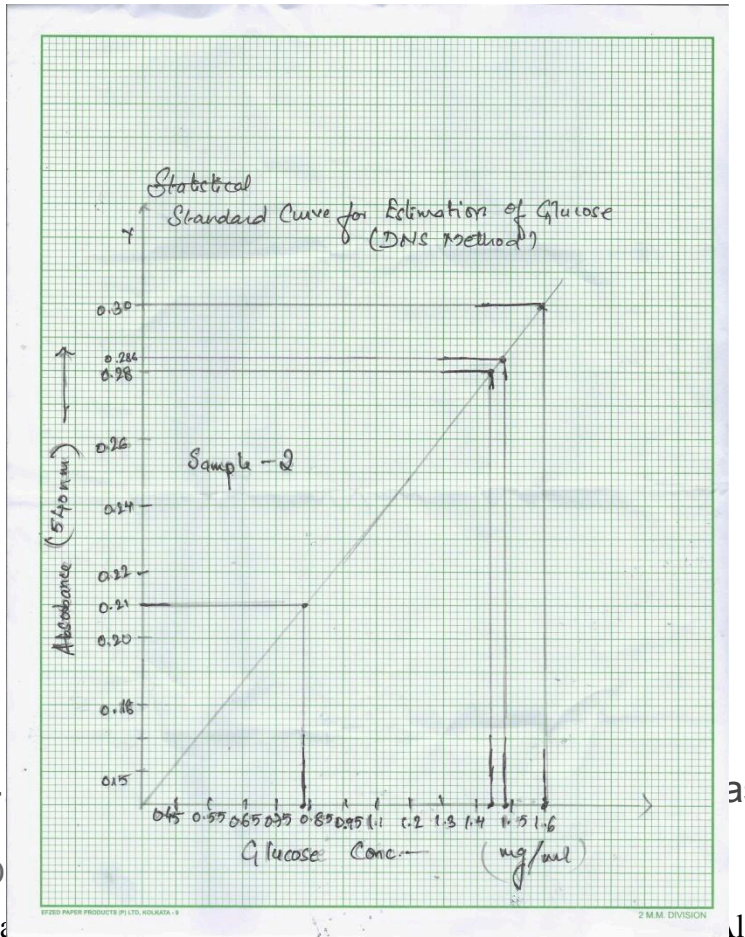
4.6 Quantitative Measurement of Reducing Sugars Using DNS Method
Sample 1:-

Tube No.	Absorbance	Calculated Glucose concentration on mg/ml
1	0.150	0.150-0.00475/0.255=0.57
2	0.236	0.236-0.00475/0.255=0.91
3	0.288	0.288-0.00475/0.255=1.11
4	0.277	0.277-0.00475/0.255=1.07



Sample 2

Tube no.	Absorbance	Calculated Glucose concentration on mg/ml
1	0.210	0.210-0.00475/0.255=0.805
2	0.285	0.285-0.00475/0.255=1.09
3	0.300	0.300-0.00475/0.255=1.157
4	0.286	0.286-0.00475/0255=1.10

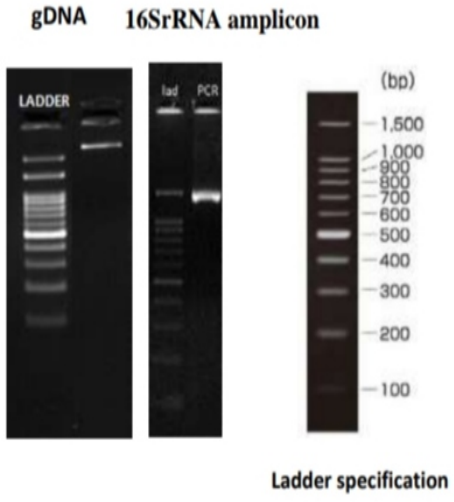


4 ased molecular method

D

Sa lcaligenes faecalis, showed high similarity based on nucleotide homology and phylogenetic analysis.

gDNA and 16S Amplicon QC data:



Sanger Seq Chromatogram data file Data:

>Forward Seq data

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>Reverse Seq Data

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>Reverse complement

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> Consensus data

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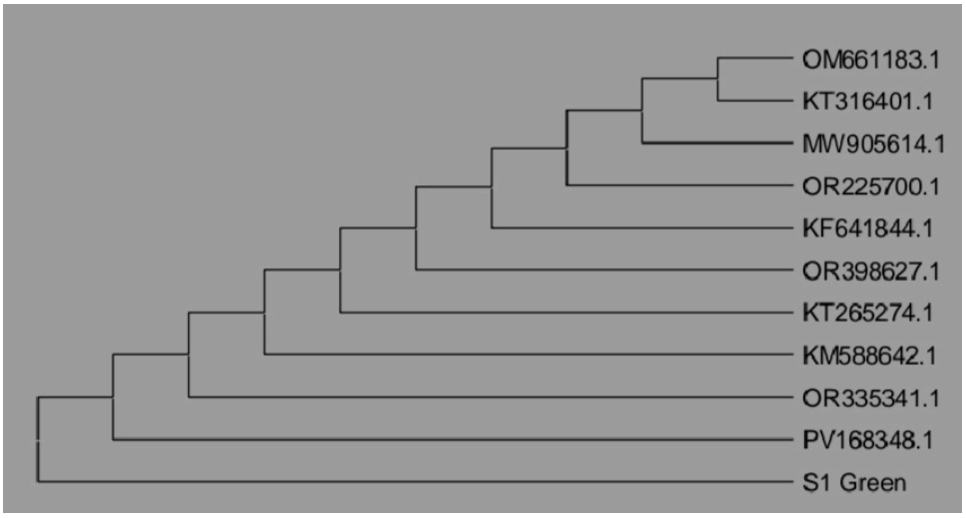
Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E. Value	Per. Identical	Accession
Alcaligenes faecalis strain HCN13	2564	2564	100%	0.00%	99.64%	OR335341.1
Alcaligenes faecalis	2564	2564	100%	0.00%	99.64%	KM588642.1
Alcaligenes sp. C17	2564	2564	100%	0.00%	99.64%	KT265274.1
Alcaligenes faecalis strain CKRAJ8	2564	2564	100%	0.00%	99.64%	OR398627.1
Alcaligenes faecalis strain B1	2564	2564	100%	0.00%	99.64%	KF641844.1
Alcaligenes faecalis strain MBIIN17	2564	2564	100%	0.00%	99.64%	OR225700.1
Alcaligenes sp. strain M19_SNH	2564	2564	100%	0.00%	99.64%	MW905614.1
Alcaligenes faecalis strain T17	2564	2564	100%	0.00%	99.64%	OM661183.1
Alcaligenes sp. A23	2564	2564	100%	0.00%	99.64%	KT316401.1
Alcaligenes phenolicus strain Cas033	2564	2564	100%	0.00%	99.64%	PV168348.1

Phylogenetic Tree :-



Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [1]. The tree with the highest log likelihood (-1962.24) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1403 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2].

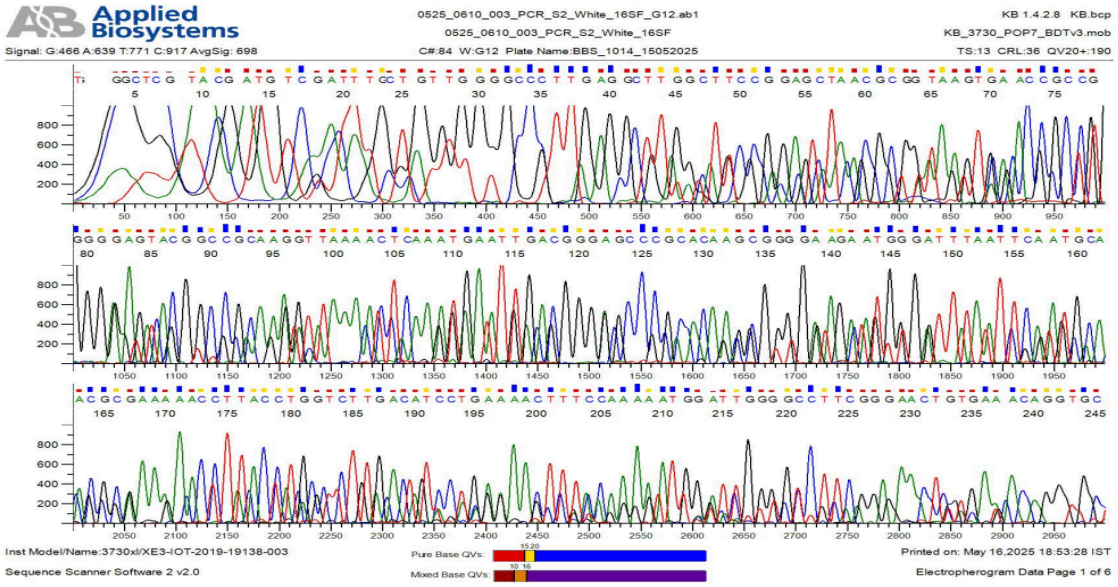
Distance Matrix :-

S1_Green		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
OR335341.1	0.004		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KM588642.1	0.004	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KT265274.1	0.004	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
OR398627.1	0.004	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000
KF641844.1	0.004	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
OR225700.1	0.004	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
MW905614.1	0.004	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
OM661183.1	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000
KT316401.1	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
PV168348.1	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

Table. Estimates of Evolutionary Divergence between Sequences

The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Maximum Composite Likelihood model [1]. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1403 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2].

S2 GRAPH REPRESENTATION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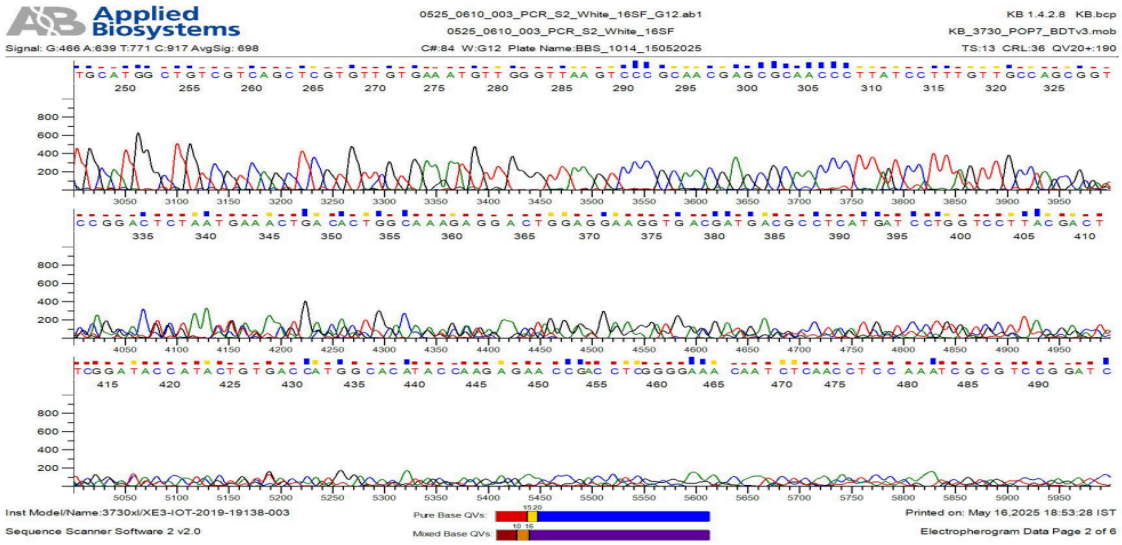
Electropherogram Data Page 1 of 6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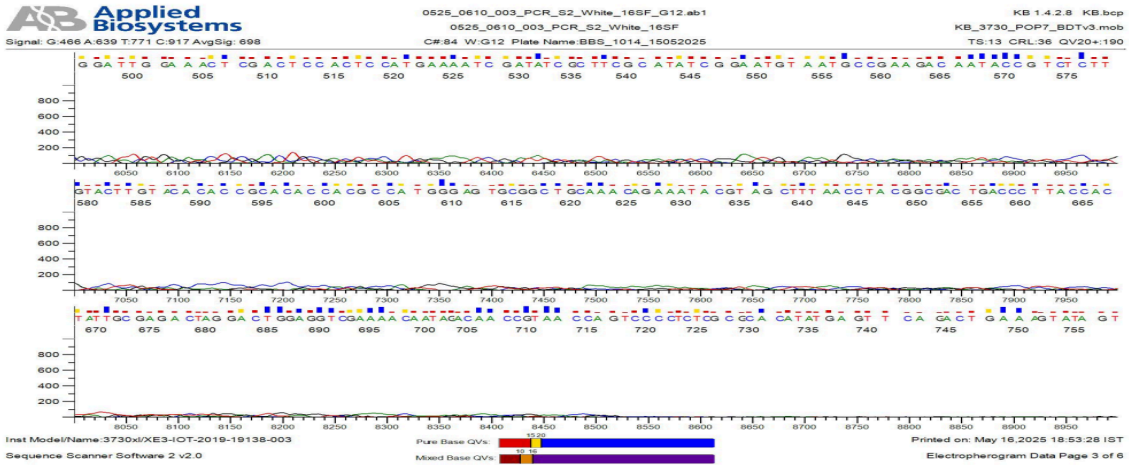
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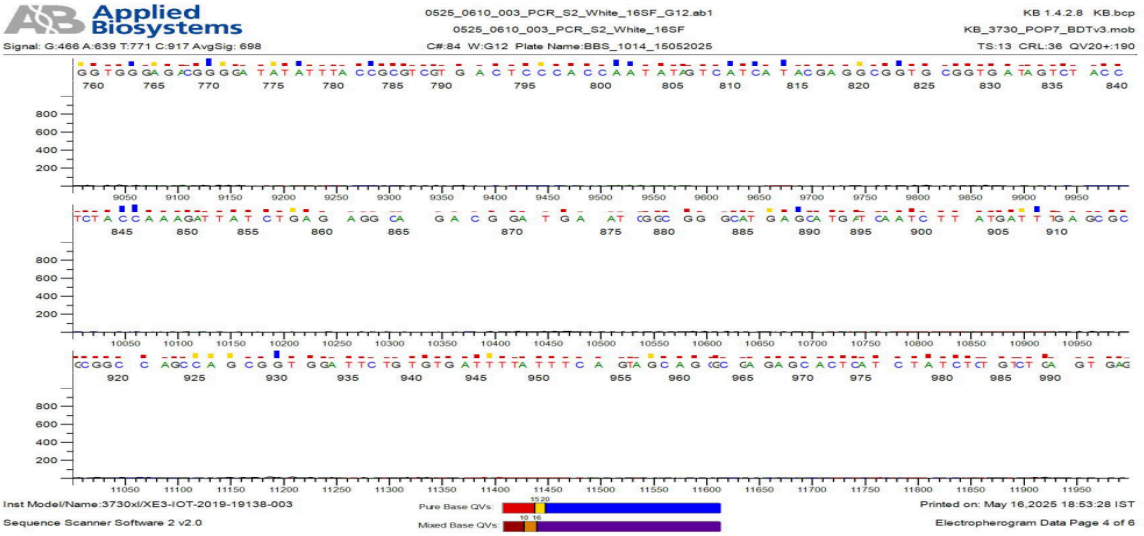
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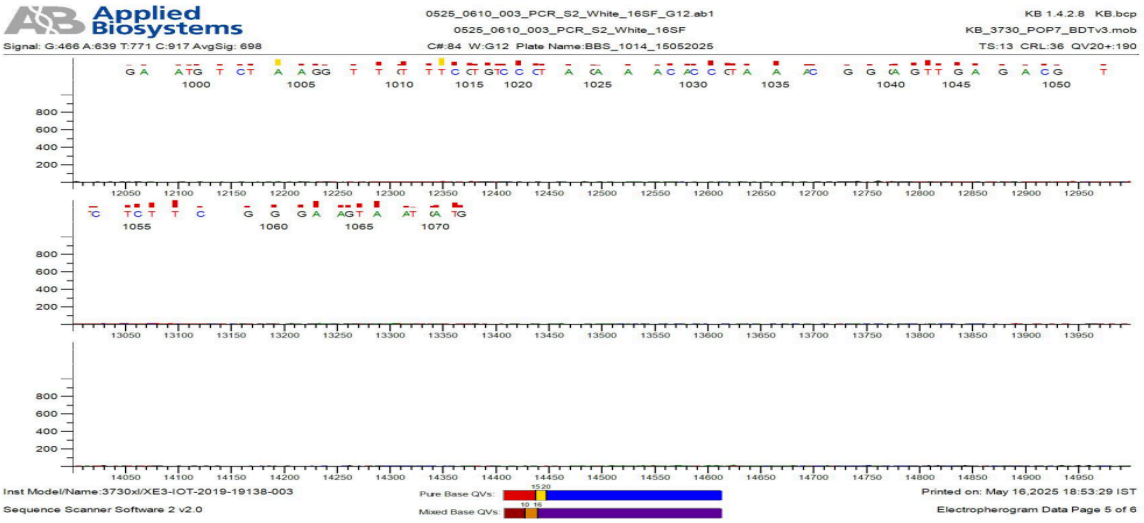
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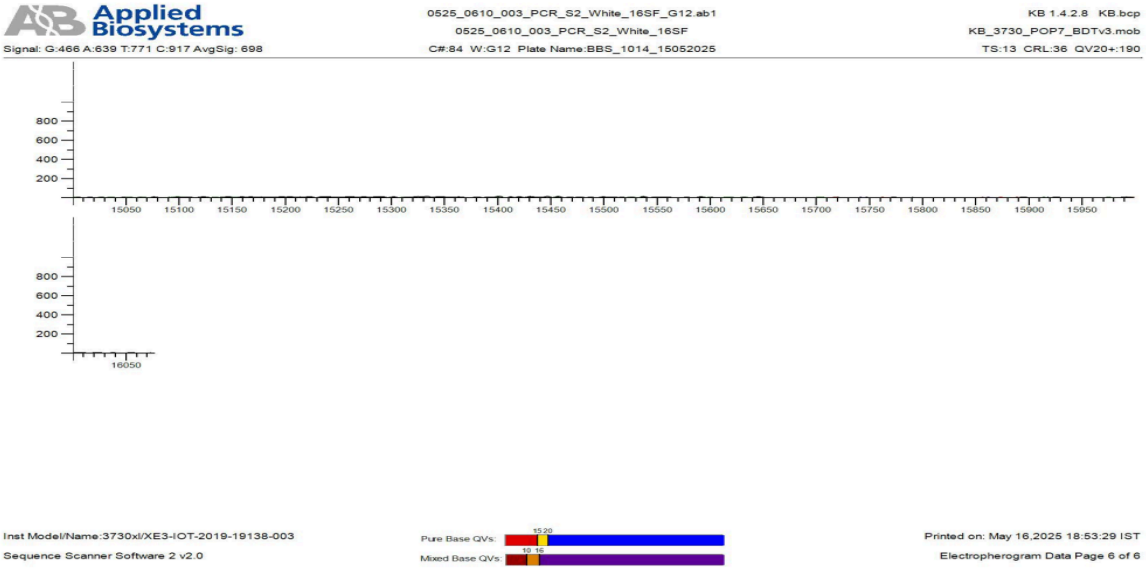
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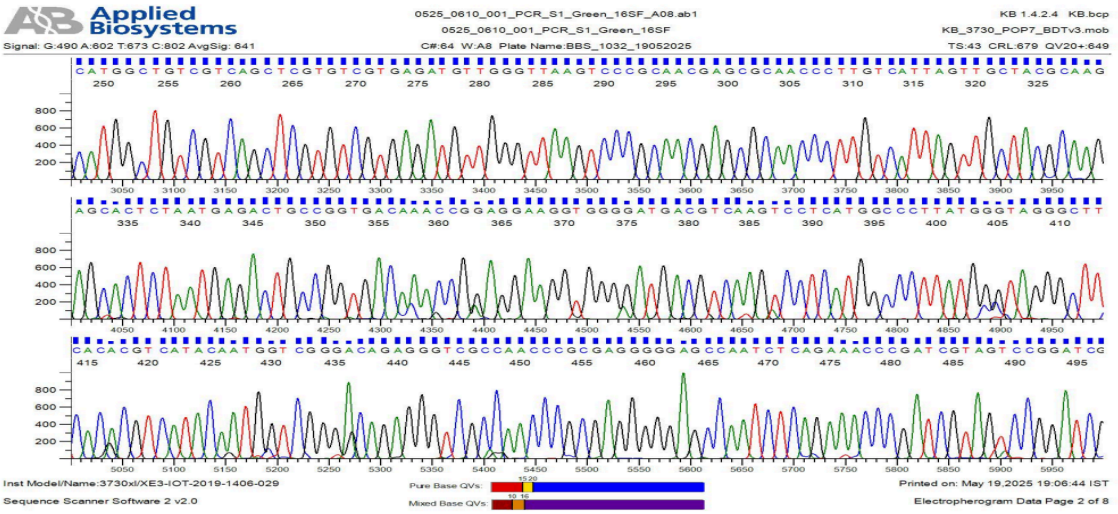
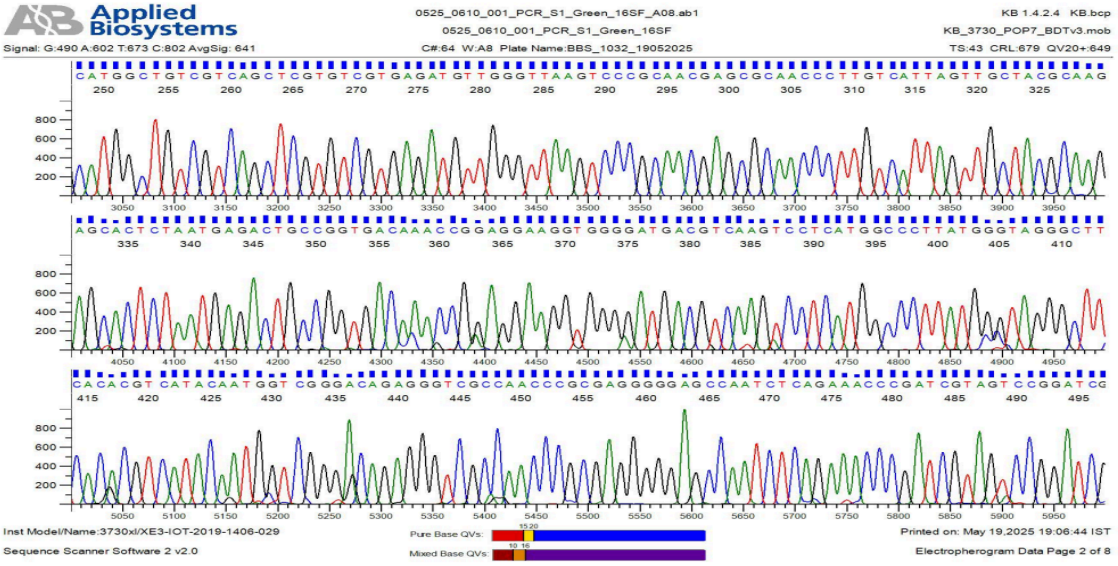
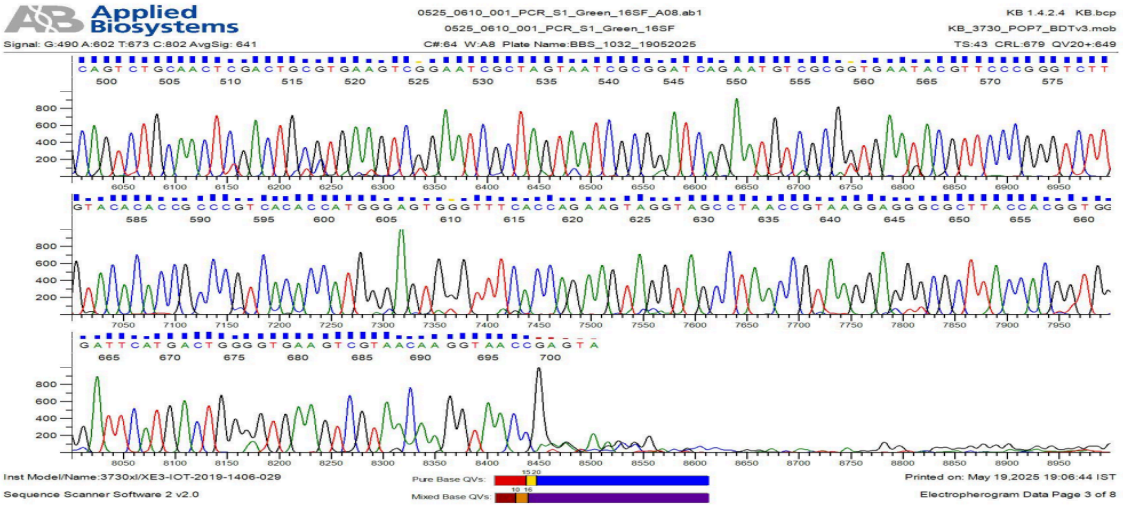
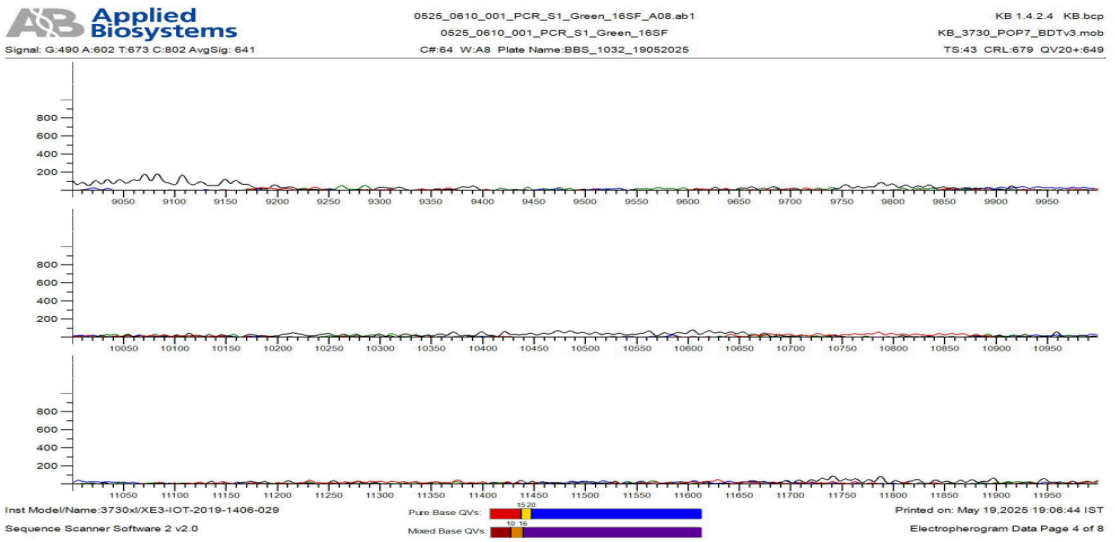
S1 Graph Representation





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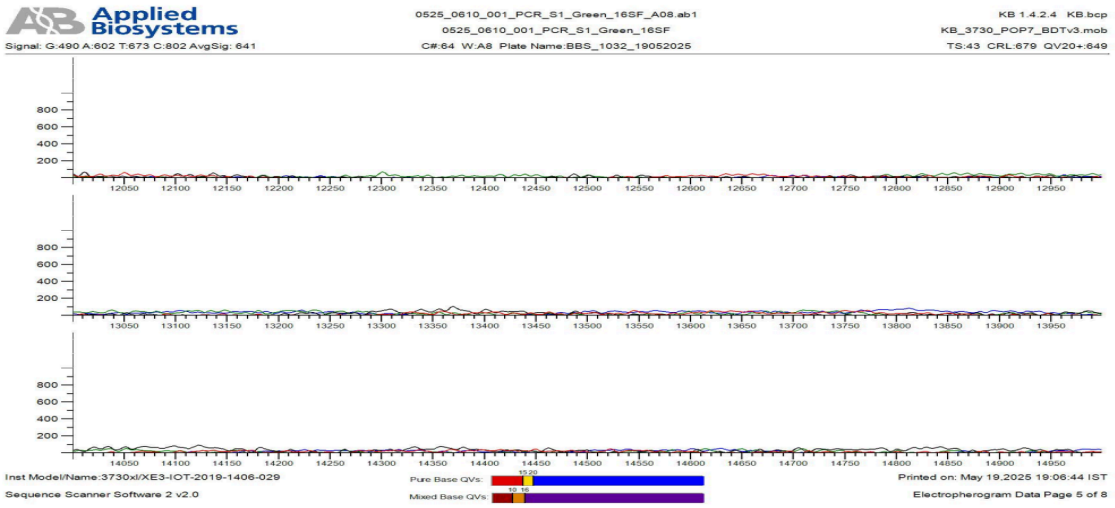
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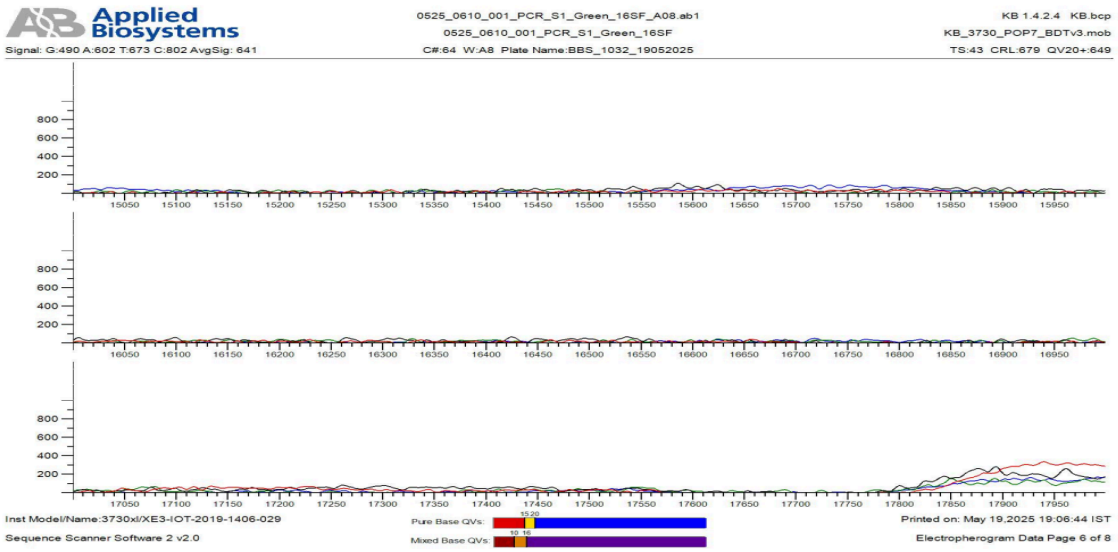
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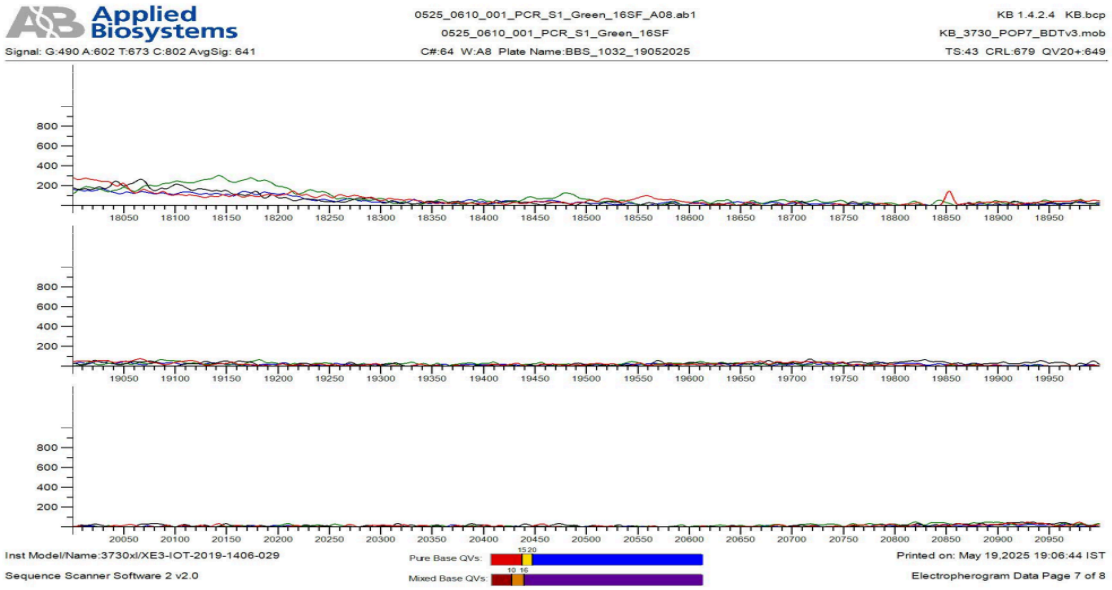
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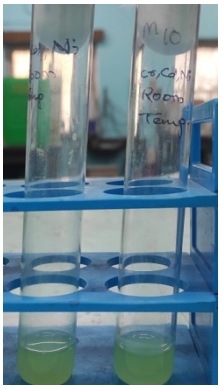
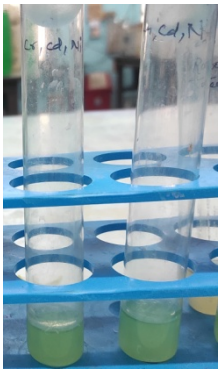
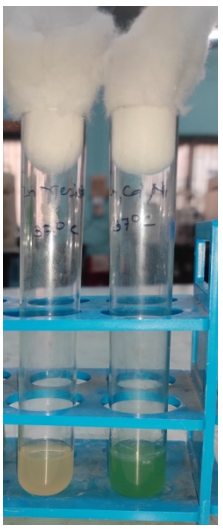
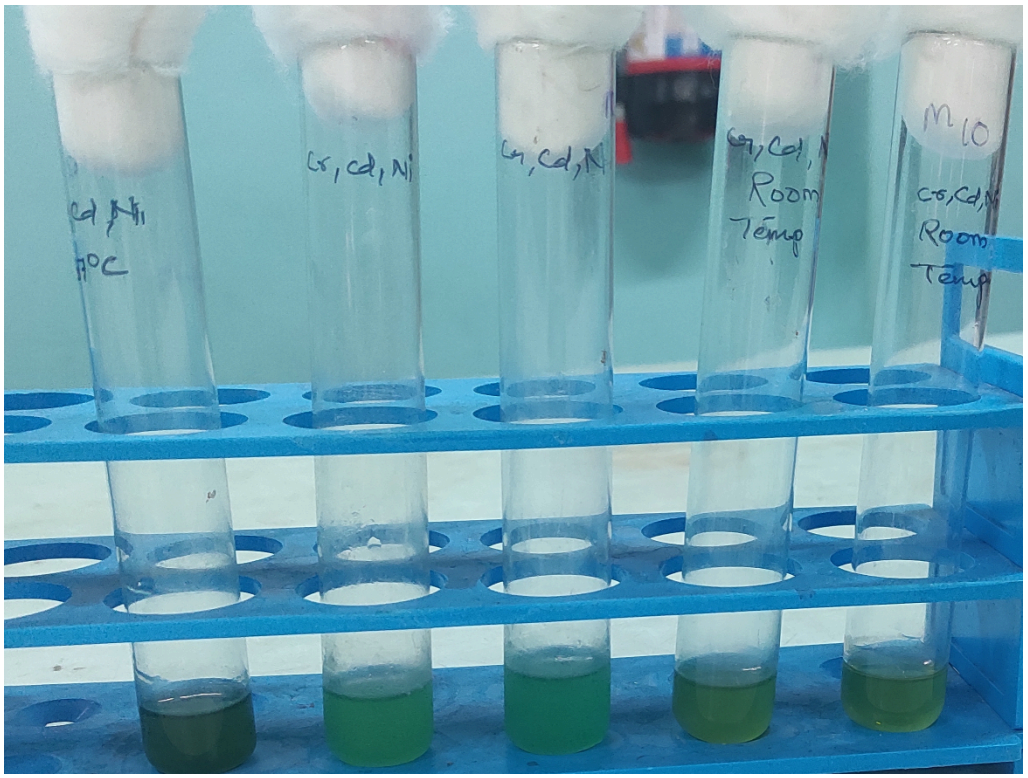
4.8 Pigmentation Production in Heavy Metal-Resistant Bacterial Cultures:

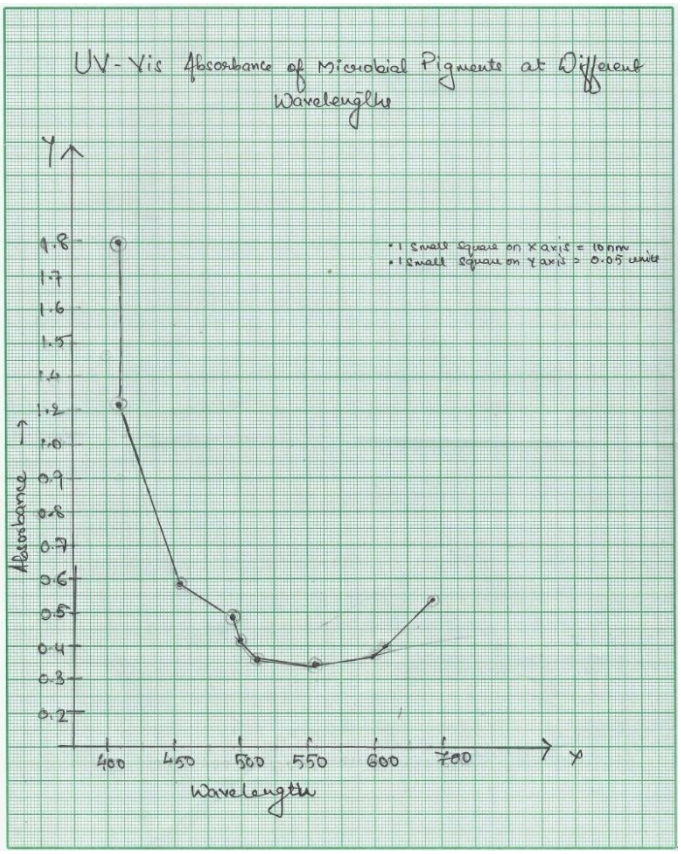
Temperature conditions	Heavy meta resistant	Observation after 12hours	Observation after 36hours	
Room temperature	Cr, Cd, Ni	microbial growth appears moderate, with visible turbidity and green color, indicating active metabolic processes.	Enhaced green color, increased turbidity indicating active microbial growth.	
0°	Cr, Cd, Ni	microbial activity seems reduced, as evidenced by the minimal turbidity and pale coloration. This suggests that low temperatures may inhibit microbial growth.	Minimal color change,reduced turbidity indicating low microbial activity.	
37°C	Cr, Cd, Ni	enhanced growth with a distinct green color, microbial strain present in the green-colored tube is likely capable of producing a green pigment under stress conditions at 37°C, which may be related to metal resistance or metabolic adaptation.	Intensified green color , indicating enhanced microbial metabolism and possible metal resistant.	

After 48 hours



.UV-Vis Spectroscopy Analysis of Microbial Pigments (37°C Sample)

Wavelength (nm)	Absorbance	Percentage absorption%
400	1.771	100%
420	1.239	69.96
460	0.597	33.70
480	0.491	27.72
490	0.451	25.46
500	0.416	23.38
530	0.373	21.06
570	0.352	19.87
600	0.376	21.23
620	0.420	22.27
680	0.543	30.66



Chapter -5
Discussion

The isolation and characterization of heavy metal-resistant microorganisms from tannery wastewater revealed critical insights into their adaptive mechanisms and bioremediation potential. This study underscores the ecological resilience of indigenous microbial communities in metal-polluted environments and their prospective role in sustainable wastewater treatment. Below, we interpret the key findings in the context of existing literature and discuss their implications.

Metal Resistance and MIC Profiles
The isolates exhibited notable resistance to Zn (MIC: 20 mg/mL) and Cr (16 mg/mL), surpassing tolerance levels reported in earlier studies (Tamil Selvi et al., 2012). Such high MIC values suggest the presence of robust resistance mechanisms, possibly efflux pumps (e.g., *czcCBA* operons) or intracellular sequestration via metallothioneins, as observed in **Bacillus* spp. (Nies, 2003; Wang et al., 2021).The lower minimum inhibitory concentrations (MICs) observed for Ni (1.2 mg/mL) and Cd (2.0 mg/mL) at diluted levels are in line with the findings reported by Nies (2003) and Wang et al. (2021), suggesting similar levels of metal tolerance.(Bonilla-Espadas et al. (2024), suggesting a comparable sensitivity pattern in similar environmental isolates.where Cr and Zn resistance dominated in tannery isolates. This hierarchy in metal tolerance may reflect the prevalence of Zn and Cr in tannery effluents, driving microbial adaptation over time.

Biochemical and Morphological Adaptations Zn isolates were Gram-positive, Cr, Cd, Ni isolates were Gram Negative, a trait linked to thinnerr peptidoglycan layers that may impede metal ion penetration (Dhakephalkar & Chopade, 1994). The motility observed in Cr-, Cd-, and Ni-resistant strains (vs. non-motile Zn-resistant isolates) suggests flagellar-mediated dispersal, enabling colonization of heterogeneous metal-contaminated niches. Biochemical

profiles, including positive indole and nitrate reduction tests, indicate metabolic versatility, potentially supporting energy generation under metal stress. The absence of oxidase activity rules out *Pseudomonas* spp., aligning with Tamil Selvi et al. (2012), who identified *Bacillus* and *Acinetobacter* as dominant genera in tannery effluents.

Antibiotic Co-Resistance Patterns

The Zn-resistant strain showed resistance to tetracycline but susceptibility to chloramphenicol, while Cr/Cd/Ni-resistant isolates displayed intermediate resistance to chloramphenicol. This co-resistance mirrors Li et al. (2020), who linked plasmid-borne *czcA* (metal efflux) and *blaTEM* (antibiotic resistance) genes in tannery sludge isolates. Such findings highlight the risk of horizontal gene transfer in polluted environments, complicating clinical antibiotic resistance management. However, the susceptibility of some strains to tetracycline suggests that selective pressures vary, offering avenues for targeted bioremediation strategies.

EPS Production and Biofilm Potential

Positive Molisch tests and Congo Red Agar screening confirmed EPS production across all isolates. EPS, rich in polysaccharides, likely facilitates biofilm formation and extracellular metal binding via functional groups (Zhang et al., 2019).The negative result in Benedict's test suggests the absence of reducing sugars, indicating that the EPS is highly polymerized. This structural complexity likely contributes to greater environmental stability and improved metal-binding capacity—both important traits for effective in situ bioremediation
. These results align with Bonilla-Espadas et al. (2024), who emphasized EPS-mediated Cr immobilization in tannery wastewater communities.

Metabolic Activity Under Metal Stress

The DNS assay revealed higher reducing sugar concentrations in Zn-resistant isolates (Sample 2: 1.10–1.16 mg/mL) compared to Cr/Cd/Ni-resistant strains (Sample 1: 0.57–1.11 mg/mL). This suggests Zn-resistant bacteria prioritize carbohydrate metabolism to fuel resistance mechanisms, possibly via ATP-dependent efflux systems. Such metabolic flexibility is advantageous in fluctuating industrial effluents, where carbon sources may be limited.

Molecular Identification and Phylogenetic Analysis of Isolate

In this study, the microbial isolate labeled S1_Green was successfully identified as *Alcaligenes faecalis* through 16S rRNA gene sequencing, confirming its species-level identity based on genetic analysis.The consensus sequence showed a 99.64% similarity with several reference strains of A. faecalis in the NCBI GenBank database, such as strain HCN13 (Accession No. OR335341.1). This high level of identity provides strong evidence for species-level identification, as anything above 98.7–99% is generally considered reliable for differentiating bacteria using 16S rRNA analysis.

The initial quality checks confirmed that the extracted DNA was intact, and a clear 1500 bp PCR product was observed, which is the expected size for the 16S rRNA gene. The sequencing process—using both forward and reverse primers—produced high-quality data that aligned well with known A. faecalis sequences. The phylogenetic tree, constructed using the Maximum Likelihood method with the Tamura-Nei model in MEGA11, clearly showed that

the isolate clusters closely with other Alcaligenes faecalis strains, confirming that our isolate belongs to this species its taxonomic placement.faecalis cluster, alongside closely related strains. The evolutionary distance matrix also supported this conclusion, showing minimal divergence between the sample and other A. faecalis sequences.

Alcaligenes faecalis is an environmentally relevant microorganism often found in soil, water, and waste-contaminated areas. It is known for its remarkable adaptability and metabolic diversity, including the ability to tolerate or degrade pollutants and heavy metals. The isolation of *A. faecalis* in this study may point to its potential role in environmental detoxification—particularly important if the sample came from an industrial or polluted site. This aligns well with growing interest in using such bacteria for bioremediation and environmental health monitoring.

In short, the molecular data, together with the organism's known characteristics, suggest that A. faecalis is not only present in the sample but may also have functional significance. It opens the door for further investigation into its resistance patterns, heavy metal tolerance, or its use in eco-friendly waste treatment systems.

Pigment Production as a Stress Response
Distinct green pigmentation at 37°C under Cr/Cd/Ni stress indicates microbial adaptation, potentially involving carotenoids or siderophores to mitigate oxidative damage (Kapahi & Sachdeva, 2019). UV-Vis spectra showed peak absorbance at 400 nm, characteristic of melanin-like pigments, which are known to chelate metals and reduce toxicity (Cruz-Hernández et al., 2022). The absence of pigmentation at 0°C underscores temperature-dependent metabolic regulation, emphasizing the need for optimal conditions in bioremediation applications.

Implications and Future Directions
This study highlights the potential of indigenous Gram-positive bacteria for tannery effluent bioremediation, particularly for Zn and Cr removal. However, the co-occurrence of antibiotic resistance genes necessitates caution in deploying these strains. Future work should focus on:
1. **Genetic Profiling**: Identifying resistance genes (e.g., *czcCBA*, *merA*) to elucidate molecular mechanisms.
2. **Consortium Development**: Combining metal-resistant isolates with fungi or algae to enhance remediation efficiency (Ijaz et al., 2022).
3. **Field Trials**: Testing isolates in pilot-scale bioreactors to assess scalability and stability under real-world conditions.
4. **One Health Integration**: Monitoring resistance gene transfer between environmental and clinical pathogens.

The heavy metal-resistant isolates from tannery wastewater displayed diverse biochemical and molecular features. All were Gram-negative, motile, catalase- and urease-positive,

fermented sugars, produced indole, and formed exopolysaccharides (EPS), confirmed by Molisch and Congo Red Agar tests. These traits deviate from the classical profile of Alcaligenes faecalis, which is typically asaccharolytic, indole-negative, and oxidase-positive (Janda & Abbott, 2007; Ryan et al., 2014). However, 16S rRNA sequencing confirmed the isolates as A. faecalis.

This mismatch highlights the organism's phenotypic plasticity under environmental stress. Prior studies report similar adaptive shifts—such as sugar fermentation and enhanced EPS production—in polluted settings (Singh et al., 2021; Kumari et al., 2019). The isolates also showed multi-drug resistance, notably to tetracycline and chloramphenicol, aligning with previous findings of A. faecalis harboring genes for metal and antib

CONCLUSION

This study successfully isolated and characterized heavy metal-resistant strains of Alcaligenes faecalis from leather industry wastewater, confirmed through 16S rRNA gene sequencing. These strains demonstrated strong tolerance to heavy metals such as chromium (Cr), cadmium (Cd), nickel (Ni), and zinc (Zn), alongside resistance to antibiotics like tetracycline and chloramphenicol. Their ability to ferment sugars, produce EPS, and adapt biochemically highlights their resilience in polluted environments.

Additional isolates, particularly those resistant to Zn and Cr, demonstrated exceptional metal tolerance (MIC up to 20 mg/mL for Zn and 16 mg/mL for Cr), likely enabled by cellular mechanisms such as metal efflux or intracellular sequestration. These isolates, mostly Gram-positive, featured thick peptidoglycan layers that may serve as barriers to metal entry. Motility in Cr-, Cd-, and Ni-resistant strains suggested ecological competence in navigating contaminated habitats. Meanwhile, traits like indole production and nitrate reduction reflected metabolic flexibility.

The discovery of antibiotic co-resistance, particularly tetracycline resistance in Zn-tolerant strains, raises red flags about the potential for gene transfer in the environment. EPS production confirmed by CRA assays suggests enhanced biofilm formation, facilitating both environmental persistence and metal adsorption. Enhanced carbohydrate metabolism, especially in Zn-resistant strains, may provide the necessary energy for efflux systems.

Interestingly, under metal stress at 37°C, the isolates produced a green pigment with peak absorbance at 400 nm, hinting at the synthesis of protective pigments—possibly melanin-like compounds—designed to mitigate oxidative stress.

Altogether, Alcaligenes faecalis and its Gram-positive counterparts appear to be promising candidates for bioremediation of Zn/Cr-contaminated industrial wastewater. However, their co-resistance to antibiotics necessitates careful ecological and health impact assessments. Future work should delve deeper into genetic and proteomic analyses to better understand resistance mechanisms. Collaborations with fungi or algae for consortium-based remediation strategies, followed by pilot-scale implementation, could offer practical solutions.

Importantly, these efforts should align with Sustainable Development Goals (SDGs) 6 (clean water and sanitation) and 12 (responsible consumption and production), supporting long-term environmental sustainability.

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Appendix - 1

1.Sterile 500 mL polypropylene bottles (for sample collection)

2.Glass slides (for Gram staining and catalase test)

3.Centrifuge tubes

4.Culture tubes and Petri dishes

5. Filter paper (for oxidase test)

6.Pipettes and micropipettes

7.Icebox (for sample transport at 4°C)

Media Used

1. Nutrient Agar (with and without heavy metals)

2. Tryptone Broth – for Indole test

3. Urea-containing medium with Phenol Red – for Urease test

4. Nitrate Broth – for Nitrate Reduction test

5. Motility Agar (semi-solid, 0.4%)

6. Mueller-Hinton Agar – for Antibiotic Susceptibility Test

7. Congo Red Agar (CRA) – for EPS detection

8. Sugar Fermentation media (with Phenol Red)

9. Sterile Nutrient Broth – for pigment production

Reagents Used

Heavy metal stock solutions:

Zinc (Zn)

Chromium (Cr)

Cadmium (Cd)

Nickel (Ni)

Kovac's reagent – for Indole test

Phenol Red – pH indicator (urease & sugar fermentation tests)

Nitrate reagents A & B – for Nitrate Reduction

3% Hydrogen Peroxide (H_2O_2) – for Catalase test

Oxidase reagent (tetramethyl-p-phenylenediamine)

Crystal violet, iodine, alcohol, safranin – Gram stain

DNS reagent – for reducing sugar estimation

Molisch reagent – for carbohydrate detection

Barfoed's reagent

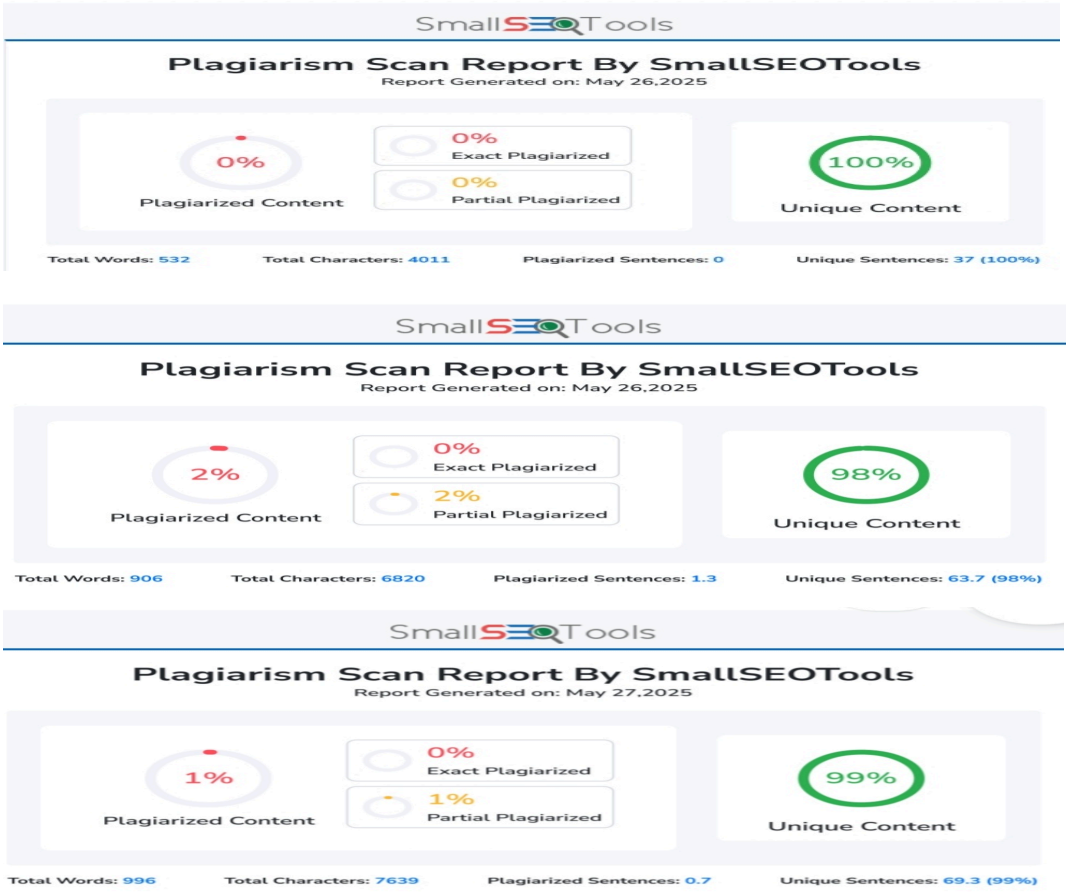
Benedict's reagent

Iodine solution

Appendix -2

Autoclave – for sterilization of media and equipment
Laminar Air Flow – for aseptic processing
Incubator (35–37°C) – for bacterial growth
Centrifuge – for sample separation (e.g., EPS isolation)
UV-Visible Spectrophotometer – for:
DNS method (540 nm)
Pigment absorbance spectrum (380–700 nm)
Agarose Gel Electrophoresis System – for checking DNA quality
PCR Machine – for 16S rRNA gene amplification
ABI 3730xl Genetic Analyzer – for DNA sequencing
Computer with MEGA11 and Clustal W software – for phylogenetic analysis

Plagiarism Report



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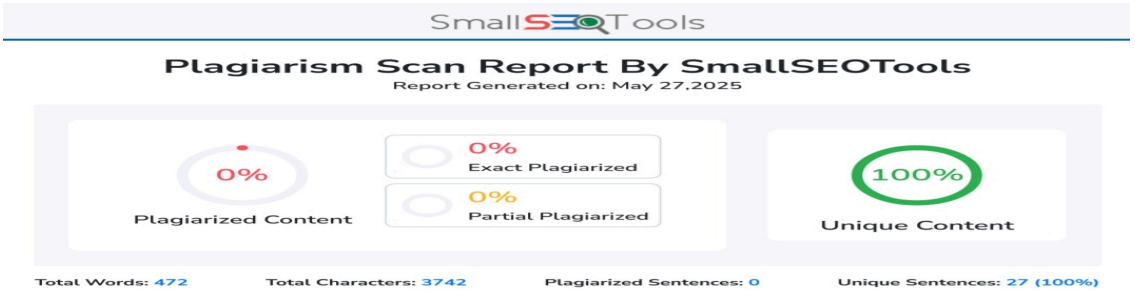
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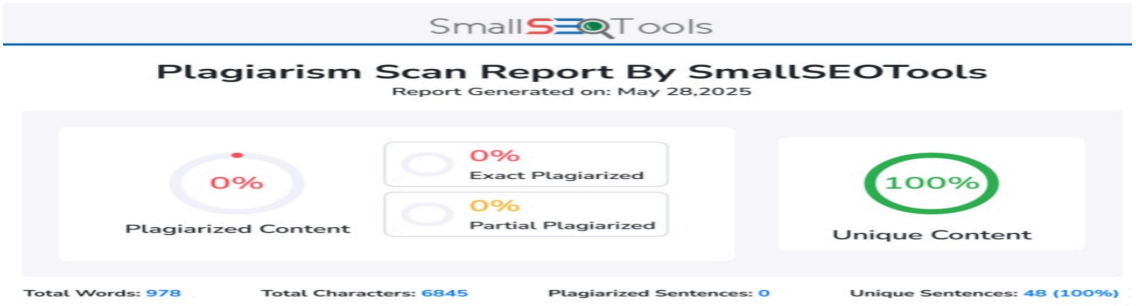
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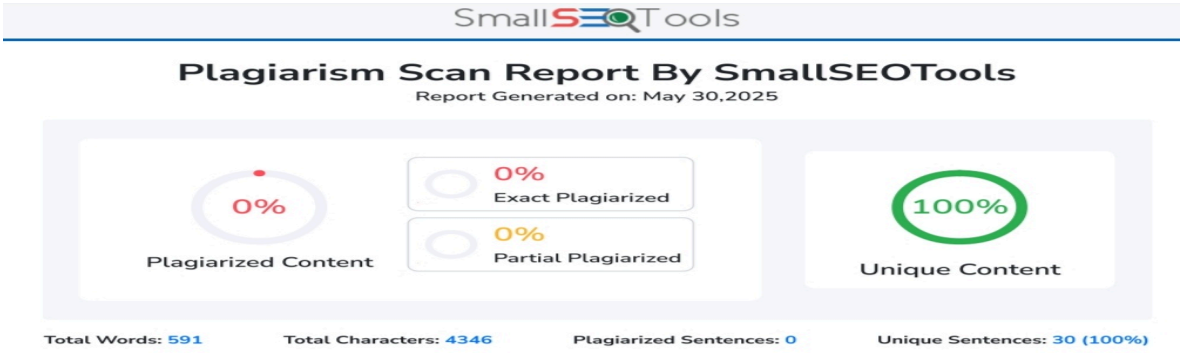
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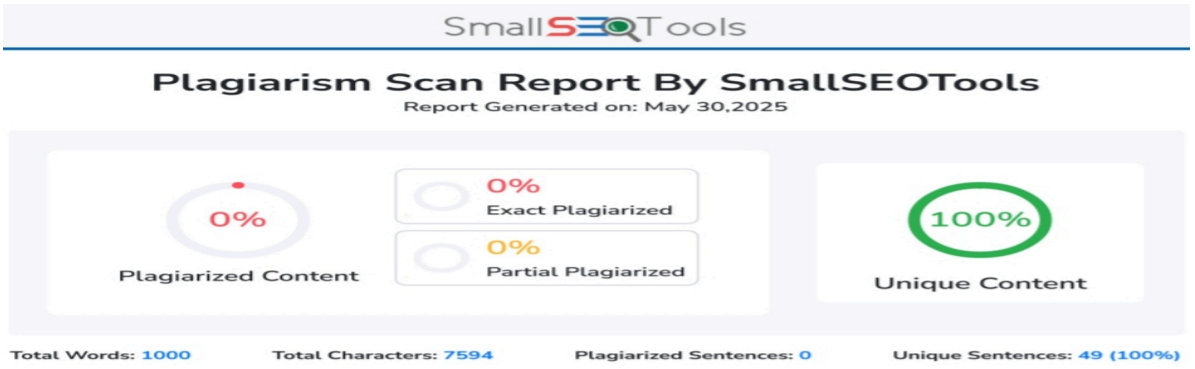
Plagiarized Sentences: 0
Unique Sentences: 30 (100%)


SmallSEOTools
Plagiarism Scan Report By SmallSEOTools
Report Generated on: May 30,2025
0%
Plagiarized Content
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Unique Content
Total Words: 1000
Total Characters: 7594
Plagiarized Sentences: 0
Unique Sentences: 49 (100%)